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Obituary Notice

FRANK LEE PYMAN, D.Sc., F.R.S.

The news of the death on 1 January 1944 of Dr Frank Lee Pyman, after a prolonged illness, at the age of 61, has been received with deep regret by his many friends in this and other countries.

Pyman was an original member of the Biochemical Society, and his death removes another member of that distinguished group of men who have been responsible for the development of chemistry—particularly medicinal chemistry—in this country during the last thirty years and to whom must be given the credit for the high respect in which British work in this field is held to-day.

Pyman was born on 8 April 1882. He acquired an early liking for chemistry at Dover College and entered Owens College, Manchester, at the age of seventeen, gaining a James Gaskell Scholarship. He graduated with first class honours in chemistry in 1902, being placed at the head of the list and awarded the Mercer Scholarship and the Le Blanc medal. He then went to the Polytechnic at Zurich to study with Bamberger, and for his research work there was awarded the degree of Ph.D. Basle in 1904. In 1910 he was awarded the degree of D.Sc. by the University of Manchester, and in 1922 (at the age of 40) he became a Fellow of the Royal Society. He was awarded the Hanbury Medal of the Pharmaceutical Society in 1936.

When Pyman returned to England from Zurich in 1905 he worked for a short time with T. E. Thorpe at the Government Laboratory, and in 1906 he was appointed to the staff of the Wellcome Chemical Works, Dartford. He followed Dr F. B. Power as Director of the Wellcome Chemical Research Laboratory in London in 1914, and in 1919 he became Professor of Technological Chemistry in the University of Manchester and Director of the Department of Applied Chemistry in the College of Technology.

In 1927 Pyman was appointed Head of the Research Department of Boots Pure Drug Company Limited in Nottingham and became a Director of the Company in 1929, which appointment he held at the time of his death.

Pyman's work falls naturally into three periods:

(1) Fourteen very productive years at Burroughs Wellcome and Company Limited, where he commenced his studies on the connexion between chemical constitution and physiological action. Important papers on tropeines, isoquinoline alkaloids, pilocarpine and glyoxaline were published. During this period Pyman collaborated with Sir Henry Dale, the late Sir Patrick Laidlaw, Dr F. H. Carr, the late Prof. Barger and Dr C. M. Wenyon and many other distinguished scientific workers.

(2) The nine years spent in Manchester widened Pyman's influence, developed his ability as a teacher and lecturer, brought him new colleagues and friends, and produced a large output of scientific work of the very highest standard.

(3) The fifteen years during which Pyman worked at Nottingham brought the influence of a distinguished academic scientific worker back to industry again, with his intellectual powers fully developed and well able to apply the scientific method to the solution of the problems of industry.

At the time of his arrival in Nottingham he was 45 years old; he soon gathered round him a group of research workers capable of following his leadership, and another very productive period followed. His paper with C. E. Coulthard and J. Marshall (*J. chem. Soc.* 1930, p. 280) on 'The variation of phenol coefficients in homologous series of phenols', arose from the work carried out in connexion with the now famous patent action between Sharpe and Dohme Inc. and Boots Pure Drug Company Limited. The experience gained in the course of this case was of the greatest value to Pyman and to his company in the years that followed.

Apart from his published work on the amidines, amoebicides and derivatives of harmine and harmaline, Pyman was able to make substantial contributions to knowledge relating to the manufacture of arsphenamines, insulin, liver extract, saccharin and potassium permanganate. When the Horticultural and Veterinary Research Departments of Boots Pure Drug Company Limited were formed, they were placed under Pyman's direction and his inspiring influence was of the greatest value, particularly during their early formative years. He had always been keenly interested in gardening and had a wide knowledge of horticultural science.

Soon after coming to Nottingham, Pyman inaugurated a monthly research conference to which distinguished academic workers were invited. His own academic background enabled him to realize the importance of not interfering in any way with the complete independence of the academic worker, and this conference functioned very efficiently and happily under his guidance.

It was characteristic of Pyman to be diffident about expressing an opinion if he thought he was

short of evidence or if he considered the problem to be unsuitable for solution by the scientific method of approach. This attitude of mind gave all the more point to his opinions when he felt inclined to express them. His dignified presence and gift of clear and concise expression inspired confidence and enabled him to put his views with great force in all circumstances.

Between 1927 and 1939 it was frequently necessary for Pyman to visit not only most of the countries in Europe but also the United States of America and Canada. Wherever he went it was evident that scientists in other countries held him in the same high regard as his colleagues at home. To be able to travel widely was a source of great satisfaction to him. He also enjoyed the opportunities it gave him to indulge his taste for good music. Few things gave him greater pleasure than an evening at the Opera House in Frankfurt, Berlin, Copenhagen, Stockholm or Paris.

Pyman was for many years a regular attender at the Annual Meetings of the Society of Chemical Industry, The British Medical Association and The British Association, and when these Societies held their meetings in Nottingham, he was called upon to play a leading part in their functions. He did so very willingly because he always appreciated the interdependence of academic science and industry.

He made friends easily and developed his capacity for friendship more and more as he grew older. All his friends and colleagues will remember gratefully the warm hospitality that he and his wife were always ready to give, and his students and all those who worked under his guidance will cherish the memory of his inspiring leadership.

L. ANDERSON

Notes on the Toxins of Egyptian Scorpions

By AHMED HASSAN MOHAMMED, *Biochemical Department, Faculty of Medicine, Fouad I University, Cairo*

(*Received* 9 *February* 1944)

This note records observations on scorpion venoms, including some details of the unpublished method of Dr & Mrs Hassan for the purification of the toxins, and an examination of the stability and absorption spectra of the purified products.

PREPARATION OF PURIFIED TOXINS

The species of scorpion used were: *Buthus quinque striatus*, *B. leptochelys*, *B. acute carinatus*, *B. hottentotta minax*. Stings and last segment of the tails (the telsons) of scorpions were detached and dried over calcium chloride. The dried telsons were ground in a mortar for about 30 min. with quartz sand, wetting with small volumes of 0·1 N-HCl. The finely ground mass was extracted with 5 × 30 ml. lots of 0·1 N-HCl, the liquid being separated by decantation. The combined extracts were neutralized with N-NaOH, a sat. aqueous solution of picric acid added and the liquid allowed to stand for 24 hr. The supernatant liquid was poured off the precipitate which had formed. This precipitate, containing the toxin, was repeatedly extracted with 80 % (v/v) acetone in water, until no more soluble picrate was removed. The toxin was then precipitated as hydrochloride by adding a few drops of conc. hydrochloric acid and an excess of acetone to the combined 80 % acetone extracts. The toxic precipitate was centrifuged off, washed successively with dry acetone and dry ether, and kept in vacuum over phosphorus pentoxide. The potency of this toxin, measured as the minimal lethal dose (M.L.D.) for the rat, was about 25–50 μg./100 g. body weight.

PROPERTIES OF PURIFIED TOXIN PREPARATIONS

In the sequel, unless otherwise stated, 'toxin' refers to a purified product prepared in the above manner. Toxin prepared as described dissolved easily in distilled water to give a clear solution. The following properties of the toxin were observed, using an aqueous solution containing 1 mg./ml.: Millon, xanthoproteic and biuret reactions were positive. There was no precipitation on boiling or half-saturation with ammonium sulphate. (Half-saturation with ammonium sulphate did produce a small flocculent precipitate, which was non-toxic, from a solution containing 4 mg. toxin/ml.) Toxin was completely precipitated by full saturation with ammonium sulphate, by absolute ethanol and by acetone. Picric acid gave a precipitate which dissolved on heating and reappeared on cooling. The general behaviour was that of a proteose. Incineration of 20 mg. of dried toxin left no residue.

Absorption spectra of toxins and venoms. Absorption spectra of toxins purified as described from the four species mentioned were examined, and also the venom from two of the species (*Buthus quinque*

striatus and *B. hottentotta minax*). Fresh venom when required was collected by the method of Jousset de Bellesme (1872), in which the gland is stimulated electrically and the venom collected through a capillary tube fitted to the sting. Venom so collected was milky in appearance, was soluble in 0·9 % saline and clotted on standing. Data on the absorption spectra are recorded in Table 1, from which it is seen that the toxins prepared from the four species all show the same absorption band at 278mμ. The two venoms both showed a band at 260mμ, the shift to 278mμ apparently occurring during the process of purification.

Table 1. *Absorption spectra data on scorpion toxins and venoms*

Species	Substance examined	Cell (mm.)	h_{max} (mμ)	E_{max}	h_{min}	E_{min}	M.L.D./100 g. rat (μg.)
B. quinque striatus	Toxin HCl 0·2 %	5	278	1·16	253	0·67	25
B. leptochelys	,,	5	278	1·44	253	0·97	33
B. acute carinatus	,,	5	278	1·43	253	0·96	27
B. hottentotta minax	,,	5	278	1·10	253	0·80	20
B. quinque striatus	Venom 0·5 ml. + 1·5 ml. saline	10	262·5	0·6	—	—	(0·3 ml.)
B. hottentotta minax	Venom 1 drop + 0·75 ml. saline	5	262·5	1·0	219	0·2	(0·1 ml.)

Effects of pH *and of temperature.* The toxins were stable when kept in solution in 0·1 N-HCl at 0° for at least 6 days, neither the absorption band nor the M.L.D. altering during this time. They were unstable in 0·1 N-NaOH, the M.L.D. being at least 10 times greater after 24 hr. at 0° and the characteristic absorption band becoming inflected. Toxin in distilled water at 55° was unaltered in 24 hr., but after 6 days had only one-tenth its former activity and the absorption band had changed.

Effect of formalin. Solutions (0·2 %) of toxin in water with different concentrations of formalin were allowed to stand at 0° and examined at intervals for toxicity and for changes in the absorption spectra. With 0·5 % formalin toxicity decreased from M.L.D. ≡ 0·05–0·08 mg. in 15 days; with 1·0 % formalin the respective values were 0·05–0·10 mg. in 15 days, without significant change in the absorption band. Even with 5 % formalin the M.L.D./100 g. rat had increased only from 0·05 to 0·16 mg. during 15 days; the absorption band was then affected.

The action of formalin at 55° was much more rapid, the M.L.D. increasing from 0·05 to 0·5 mg./100 g. rat in 24 hr. with 0·5 % formalin, or within 1 hr. with 5 % formalin, the characteristic absorption band becoming inflected.

Effect of acetaldehyde. When acetaldehyde (5 %) was used instead of the formalin at 55°, the potency of the toxin decreased from 0·05 to 0·08 mg./100 g. rat in 1 hr. without alteration of the absorption band, but after 24 hr. under these conditions the M.L.D. had increased to 0·5 mg. and the characteristic absorption band was no longer present.

It appears from the parallelism in the changes in toxicity with the changes in the absorption band produced by various treatments that this absorption band is related to the toxin.

My thanks are due to Prof. A. Hassan, Dr A. Hassan and Dr M. S. El Ridi for their interest and help.

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Analysis of a Partial Hydrolysate of Gramicidin by Partition Chromatography with Starch

By R. L. M. SYNGE, *The Lister Institute of Preventive Medicine, London*

(*Received* 6 *June* 1944)

Following the analysis of the constituent amino-acids of gramicidin previously reported (Gordon, Martin & Synge, 1943*b*), a beginning has been made with a study of the peptides arising from the partial hydrolysis of gramicidin by acid. In the present paper are described the use of a new technique (partition chromatography employing starch) which has proved valuable for this work and may have wider applicability, and the isolation of the dipeptide *l*-valylglycine from gramicidin by partial hydrolysis with acid. Some further evidence concerning the amino-acid composition of gramicidin and the optical nature of the constituent valine residues has been obtained.

COMPARISON OF AMINO-ACID COMPOSITION AND OTHER PROPERTIES OF GRAMICIDIN FROM TWO SOURCES

The Wallerstein gramicidin previously studied was insufficient for the present work; it seemed best to employ it in a detailed comparison with a new batch obtained from Dr R. D. Hotchkiss. The two specimens were compared in respect of melting-point, rotation, N content and amino-acid composition, properties which did not differ significantly from one specimen to another nor after recrystallization (Table 2). For the purposes of this discussion they are regarded as identical. It has not proved possible to ascertain whether both specimens were obtained from the same strain of organism.

In determining the amino-acid composition somewhat milder conditions of acid hydrolysis were employed than formerly (Gordon *et al.* 1943*b*), since control experiments on known amino-acid mixtures had shown (Table 1) that a better tryptophan recovery could be obtained in this way. With gramicidin itself, the tryptophan figures were indeed more accurate than those previously found, but the values for other amino-acids were consistently and significantly different from those originally published. Subsequent to these analyses, Christensen (1943) reported the occurrence of valylvaline in acid hydrolysates of gramicidin, and valylglycine has now been isolated as described below. The presence of these peptides, which are unusually resistant to acid hydrolysis, sufficiently explains the deviations from results previously obtained. It is therefore justifiable to attribute to gramicidin the composition determined after more vigorous hydrolysis (Gordon *et al.* 1943*b*), viz.: 6 leucine, 6 tryptophan, 5 valine, 3 alanine and 2 glycine residues and 2 residues of an unknown hydroxyamino compound per assumed molecule. Experiments employing the 'paper strip' partition chromatographic technique of Consden, Gordon & Martin (1944) were undertaken with gramicidin hydrolysates, in the hope of detecting this unknown compound, but strips developed with *n*-butanol-H_2O or phenol-H_2O mixtures on treating with ninhydrin revealed pictures which could be simulated in every respect by mixtures of the known components of gramicidin. (This provided an independent check on the qualitative amino-acid composition of gramicidin.) Determination of the NH_2 liberated by periodate from gramicidin hydrolysates (Table 2) confirmed Hotchkiss's (1941) results quantitatively, whereas a mixture of the known constituents of gramicidin treated in the same way yielded negligible NH_2. The reality of this hydroxyamino component is thus not in doubt, but it is possible that it is not an amino-acid at all. It is clearly important to determine the nature of this ultimate hydrolysis product before proceeding further in the study of partial hydrolysis.

TECHNIQUE OF SEPARATION OF PARTIAL HYDROLYSIS PRODUCTS

Preliminary experiments done in collaboration with Dr A. H. Gordon at Leeds showed:

(1) About half of the peptide bonds in gramicidin are split by treatment with 10N-HCl and acetic acid (1:1 v/v) at 37° for 10 days, as judged from the Van Slyke amino-N determination. All partial hydrolysates discussed here have been prepared in this way.

(2) Partition chromatography on silica gel of acetylated partial hydrolysates gave a complicated series of bands, some of which 'tailed' so excessively on the columns that separations were obviously less effective than those obtained in our study of gelatin (Gordon *et al.* 1943*c*) by this technique. Quantitative amino-acid analysis of the resulting fractions confirmed the ineffectiveness of the separations. It has already been shown (Gordon *et al.* 1944) that acetyltryptophan 'tails' on silica gel partition chromatograms, and it is to be expected that acetylpeptides containing more than one tryptophan residue would show excessive 'tailing'.

(3) On paper strip partition chromatograms (Consden *et al.* 1944) a number of free peptides travel as reasonably narrow bands whose presence can be revealed by treatment with ninhydrin. Table 3 shows the relative band-rates of some of these in the *n*-butanol-H_2O system. The 'strip' and 'two-dimensional' paper chromatograms with gramicidin partial hydrolysates revealed an array of fairly well-separated compounds greater in number, and in many cases differing in position from those obtained with complete hydrolysates.

Further experiments have now been conducted with gramicidin partial hydrolysates on *n*-butanol-H_2O paper strip chromatograms. Colouring these by spraying with Ehrlich's reagent in acid solution revealed two well-marked bands (presumably of tryptophan peptides) faster than the band corresponding to free tryptophan, but none slower than it. 'Tailing' of tryptophan-containing substances appeared to be negligible. Attempts were therefore made to reproduce this type of chromatogram on the preparative scale.

Use of starch chromatograms. Working with Dr S. R. Elsden, and on his suggestion, we employed raw potato starch, and found it entirely suitable for the purpose (Elsden & Synge, 1944). It was found that amino-acids travel as quite sharp bands in column chromatograms made up with starch and developed with *n*-butanol saturated with water. The presence of the amino-acid bands was revealed by passing an ethereal solution of ninhydrin through the column after development (Consden *et al.* 1944).

Comparison of the partition coefficients of simple monoamino-acids as calculated from their band-rates and the chromatogram constants (Table 4)

shows fair agreement with the direct determinations of England & Cohn (1935). The calculations are similar to those of Consden *et al.* for paper chromatograms, and suggest that the phenomena are essentially the same, as would be expected on chemical grounds. In practice it was found that gramicidin partial hydrolysates gave an identical sequence of bands (examined by fluorescence, ninhydrin and Ehrlich's reagent) on paper or starch chromatograms, and it seems probable that most of the separations described by Consden *et al.* will be reproducible on the preparative scale using starch. Preliminary experiments have shown that the glutamic acid-aspartic acid separation can be done on starch as well as on paper with phenol-H_2O.

There has been no indication in these experiments that optical antipodes or racemates of any compound travel at different rates on the chromatograms. Resolution of optical antipodes is a theoretical possibility on account of the asymmetry of starch and cellulose; that it does not occur provides further evidence that the latter substances act as inert supports for a liquid phase.

The experience with gramicidin partial hydrolysates on starch chromatograms is discussed later. Here it is only necessary to draw attention to the fact that when this mixture was applied as the hydrochlorides of its components, valylglycine travelled as a band (as hydrochloride) at about twice the rate of alanine (which emerged from the chromatogram almost free from HCl). These two substances in the free state travel as bands having closely similar rates in *n*-butanol-H_2O and would be difficult to separate. The presence of HCl apparently exerts a differential effect. This observation has not yet been followed up, but taken with the data of Consden *et al.* on the effects of varying pH, it suggests that new and useful separations may be effected by control of this factor.

Throughout this work the qualitative ultra-micro paper-strip techniques described by Consden *et al.* have proved very useful for controlling analytical operations, checking purity, and confirming identity while sacrificing only a few μg. of material.

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF GRAMICIDIN PARTIAL HYDROLYSATES

It was found that the colour reaction with ninhydrin in ether on starch chromatograms was useful for distinguishing free amino-acids from peptides, which gave no colour until heated. Partial hydrolysates of gramicidin, in contrast to complete hydrolysates, did not give bands corresponding to free valine or glycine. The leucine band, the tryptophan band (slightly behind the leucine and imperfectly separated from it) and the alanine band were always observed. The absence of free valine is confirmed by the quantitative analyses given below. The absence of these amino-acids in the free state fits in with the finding of large yields of peptides embodying them both by Christensen (1943) and in the present work.

After pilot chromatograms had been studied, a large-scale chromatographic analysis was done on a starch chromatogram employing *n*-butanol-H_2O as developing solvent. Fractions were collected 'blind' from the effluent in pre-arranged volumes, but examination of the fluorescent bands of tryptophan peptides, etc., under ultra-violet irradiation showed that flow was quite even and that all parts of each band-front arrived at the bottom of the chromatogram simultaneously.

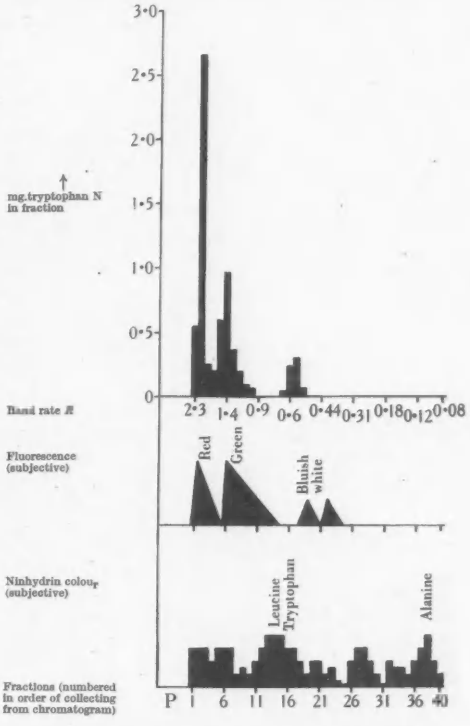

Fig. 1. Properties of fractions of gramicidin partial hydrolysate.

The 40 fractions collected were analyzed for tryptophan by a modification of the method of May & Rose (1922), and estimates of fluorescence and the colour on heating with ninhydrin were also made. Results are illustrated in Fig. 1. No tryptophan peptides appeared to move more slowly than free tryptophan, and the present paper deals with

the bands slower than, and free from tryptophan, which represent a small, relatively simple portion of the mixture. The faster moving components await further investigation.

No material moving more slowly than alanine and giving colour with ninhydrin had been detected in the preliminary experiments, so the large-scale chromatogram was discontinued when the alanine band had run out.

Small samples of the fractions following the tryptophan were hydrolyzed with acid, and the constituent amino-acids determined qualitatively on paper-strip chromatograms, with the results shown in Fig. 2. On the basis of this information, the fractions were placed in five groups (*A*, *B*, *C*, *D*, *E*, Fig. 2) and bulked samples from all the fractions in

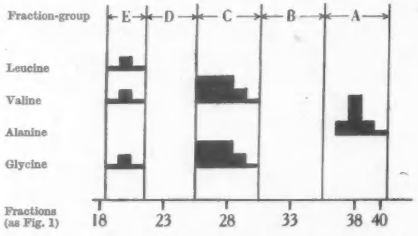

Fig. 2. Amino-acid composition and grouping of slower-moving fractions.

each group were hydrolyzed and subjected to quantitative amino-acid analysis (Table 5). Groups *B* and *D* contained negligible quantities. The other groups are discussed below.

Group A. That this fraction consisted mainly of *free alanine* is established by (1) its band-rate on the starch chromatograms and its reaction with ninhydrin in the cold; (2) qualitative (on paper) and quantitative (on silica gel) analyses of its constituent amino-acids after hydrolysis; (3) the fact that direct acetylation of the material in this fraction gave a good yield (on the alanine content of the fraction) of material agreeing in properties with acetylalanine and yielding on hydrolysis nearly one molecule of alanine per —COOH group. Were a peptide such as alanylalanine present, hydrolysis of its acetyl derivative would yield two or more molecules of alanine per —COOH group.

Group C. This fraction consisted mainly of l-*valylglycine*. For the quantitative analysis of its amino-acid composition, hydrolysis by 6N-HCl at 100° for 24 hr. was employed. The resulting analysis, in addition to equimolar quantities of acetylglycine and acetylvaline, gave a strong additional band agreeing in rate with acetylalanine. Since, however, the strip experiments excluded the presence of this quantity of alanine, it seemed possible that the acetyl derivative of unhydrolyzed dipeptide might be present. Glycyl-*dl*-valine and *dl*-valylglycine were prepared and subjected to the same conditions of hydrolysis and subsequent analysis. It was found that while glycylvaline gave the expected yield of valine and glycine only, valylglycine gave a result corresponding qualitatively and *quantitatively* with that from the natural product. It was then established that the natural product: (1) on electrometric titration agreed quantitatively in behaviour with valylglycine; (2) on acetylation gave a good yield of material agreeing in chromatogram band-rate with acetylvalylglycine, having the expected acid equivalent weight, and yielding on hydrolysis one molecule of valine and one of glycine per —COOH group; (3) had a strong positive optical rotation consistent with that found by Fischer & Scheibler (1908*a*) for synthetic *l*-valylglycine, and on acid hydrolysis yielded *l*-valine of high optical activity (as found by Fischer & Scheibler (1908*a*) with the synthetic material). This third observation provides additional evidence for the order of the amino-acid residues, since Fischer & Scheibler (1908*a*) found that synthetic glycyl-*l*-valine has a negative rotation.

The techniques employed for establishing the structure of the compound were chosen to avoid destruction of the component residues, since it was desired to conserve as much of the valine as possible for the determination of its optical form. It was further necessary to avoid all fractionation depending on crystallization, in order to conserve optical character. Chromatographic purifications were therefore employed throughout.

Group E. Similar evidence to that adduced in the previous section suggests that the 'alanine' of the amino-acid analysis of this fraction is an artefact of incomplete hydrolysis. The material may contain a tripeptide embodying leucine, valine and glycine, but the total amount so far available has not permitted more detailed study.

From these experiments it is apparent that the partial hydrolysate yields about 0·75 mol. of free alanine per assumed gramicidin molecule, and about 1·15 mol. of *l*-valylglycine, or more if it be assumed that fraction *E* embodies this peptide attached to leucine at one or other end. It is therefore probable that both the glycine residues of the assumed molecule occur linked through their $—NH_2$ groups to *l*-valine. This result serves as a sort of control of the 'partial hydrolysis' approach to molecular structure. Gordon *et al.* (1941) stressed the desirability of obtaining a check of this kind on the method.

The high yield of *l*-valylglycine is readily explained by the resistance of *dl*-valylglycine to acid hydrolysis whilst Christensen's (1943) isolation of valylvaline suggests that valyl peptides as a class may be particularly resistant. Fischer & Abderhalden (1907), moreover, obtained evidence for the occurrence of a dipeptide of glycine and valine in

partial acid hydrolysates of elastin. The study by Levene, Steiger & Rothen (1932) of the hydrolysis of a number of dipeptides by alkali at 37° showed valylglycine to be the most resistant of these. No systematic experiments on the kinetics of acid hydrolysis of simple peptides seem to have been published. It is hoped shortly to undertake such a study in relation to the kinetics of acid hydrolysis of gramicidin. The results so far available, together with Steiger's (1934*b*) study of the hydrolysis of a number of acetyl peptides by the acidity of their own aqueous solutions, suggest considerable parallelism between the resistance of peptides of aliphatic monoamino-acids to acid and to alkali. If this be established, it will be unfortunate for partial hydrolysis studies, since alkali will give breakdown products similar to those obtained with acid.

THE OPTICAL CHARACTER OF THE VALINE RESIDUES IN GRAMICIDIN

Gordon *et al.* (1943*b*) suggested two possible explanations for the racemic character of the valine isolated from acid hydrolysates of gramicidin, and the isolation of *l*-valylglycine from partial hydrolysates of gramicidin now permits a hoped-for experimental approach to this problem. (In the following discussion the possibility of Walden inversion during hydrolysis, acetylation, etc., is ignored, since it seems unlikely that it can occur. It should be noted, however, that Fischer & Scheibler (1908*b*) have described some anomalous behaviour by valine derivatives in respect of Walden inversion.)

On the evidence now available it appears that in the gramicidin molecule two *l*-valine residues occur linked through their carboxyl groups to glycine, and further that on acid hydrolysis for 10 days at 37°, followed by that for 36 hr. at 115°, these will give nearly 2 mol. of *l*-valine in the hydrolysate.

The optical activity of the valine resulting from gramicidin hydrolyzed in this way was determined by acetylating the hydrolysate, isolating the valine chromatographically as its acetyl derivative, hydrolyzing this fraction, and determining its rotation in 6N-HCl. By eliminating crystallization, fractionation of optical isomers was avoided. The only known optically active contaminant, *l*-tryptophan, has a negligible rotation in HCl (Abderhalden & Kempe, 1907*a*; Lutz & Jirgensons, 1931). The valine resulting was found to have $(\alpha)_D$, $-10° \pm 3°$. *l*-Valine has $(\alpha)_D$, $+28{\cdot}8°$ (Fischer, 1906).

Since two of the five valine molecules obtained in this way must be *l*-valine, it is necessary to postulate that the other three are *d*-valine and thus evidence is provided for the co-existence at different points in the gramicidin molecule of both *d*- and *l*-valine residues.

When the gramicidin was hydrolyzed at 115° without preliminary treatment at 37°, the valine isolated in the same way had $(\alpha)_D$, $0° \pm 3°$. This suggests that racemization may result from the use of hot acid in the early stages of hydrolysis. Racemization of residues in peptide linkage by hot acid has been demonstrated by Levene & Steiger (1930), and this seems the most plausible explanation of the racemic character of the valine obtained by Christensen (1943) from valylvaline resulting from *hot* acid hydrolysis of gramicidin.*

It is therefore to be expected that peptides embodying *d*-valine will be found on further investigation of partial hydrolysates of gramicidin. In their preparation, hot acid should be avoided in the early stages of hydrolysis.

Gramicidin seems to be the first protein-like material with which there is evidence for the occurrence of both *d*- and *l*- residues of the same amino-acid in the molecule. To demonstrate this conclusively the isolation of peptides of *d*-valine will be necessary. In other cases, where partially or completely racemic amino-acids have been reported from acid hydrolysates of proteins (e.g. Chibnall, Rees, Williams & Boyland (1940); Martin & Synge (1941*a*)), it seems preferable to continue to regard these as arising by selective racemization of the *l*-isomer during hydrolysis until evidence to the contrary is advanced, e.g. by isolation of partial hydrolysis products. There is some evidence that selective racemization of this kind occurs in the early stages of hydrolysis of gramicidin by hot acid.

EXPERIMENTAL

Methods

Partition chromatography of acetyl derivatives on silica gel. Batch *γ* of silica gel (Gordon *et al.* 1944) was employed throughout this work. Unless otherwise stated, material was acetylated and prepared for chromatography according to Gordon *et al.* (1943*a*); 3 g. silica gel were made up in columns having 1 cm. internal diameter. Peonin chloride (Gordon *et al.* 1944) was employed as indicator for all chromatograms except those initially made up with 3% *n*-butanol-chloroform; in these the '*R*-NH_4' indicator described by Liddell & Rydon (1944) was used.

The 'separating funnel' extraction procedure in preparing material for chromatography was retained for rapidity of working in spite of selective losses of alanine and glycine and some inaccuracy, which could have been obviated by using a 'column' extraction procedure (Gordon *et al.* 1943*d*, 1944). In control experiments, recoveries of alanine have averaged 96%, of glycine 75%, and the figures for these acids given below have been corrected accordingly. Chro-

* (*Note added in proof.*) H. N. Christensen (*J. biol. Chem.* **154**, 427 (1944)), has now shown that the valylvaline isolated by him is the *dd-ll* and not the *dl-ld* racemic modification. This cannot, he considers, arise by racemization during hydrolysis. Nevertheless, it might be that steric factors necessitate the racemization of a -*d*-valyl-*d*-valyl- grouping by epimerization at both asymmetric centres simultaneously or not at all.

matographic manipulation and titration of the resulting fractions was as described by Gordon *et al.* (1943*a*).

Partition chromatography of free amino-acids and peptides on filter-paper strips. This was done according to the general directions of Consden *et al.* (1944). Where material was applied to the paper as its hydrochloride, it was not neutralized with NH_3 before development of the chromatogram. Whatman no. 1 filter paper was employed throughout.

Miscellaneous. Optical rotations were determined in a 0·5 dm. tube. Melting-points are recorded uncorrected. All organic solvents were redistilled in the laboratory through a fractionating column. Glacial acetic acid was distilled from $K_2Cr_2O_7$ at atmospheric pressure. Unless otherwise stated, evaporations were *in vacuo* below 40°.

Preparation of peptides, etc. studied

dl-*Valylglycine* was prepared according to Fischer & Schenkel (1907). Satisfactory amination was not achieved by following the directions of Levene *et al.* (1932).

Glycyl-dl-*valine* was prepared according to Levene *et al.* (1932). In contrast to their data, the air-dry product lost only 2·8% of its weight on drying to constant weight over P_2O_5 at 100°. There is thus little evidence for water of crystallization; Abderhalden, Rindtorff & Schmitz (1928) noticed none.

dl-3-iso*Propylpiperazine*-2:5-*dione* was prepared according to Fischer & Schenkel (1907) from *dl*-valylglycine. The product had m.p. 244–245°.

l-*Leucyl*-l-*tryptophan* was prepared (with Dr A. H. Gordon) according to Abderhalden & Kempe (1907*b*).

Glycylglycine, *glycyl*-dl-*leucine*, dl-*leucylglycine* and dl-*leucyl*-dl-*leucine* 'A' were the preparations referred to by Gordon *et al.* (1943*a*). d-*Leucylglycylglycine* was obtained from Roche Products Ltd.

Preparation of gramicidin specimens studied

(1) *Material received from Dr R. D. Hotchkiss.* The material (R1) as received had not been recrystallized. Recrystallization from acetone, followed by washing of the product with pure ether (British Pharmacopoeia), yielded R2, 21% of material passing into the mother liquors. R3 was obtained in the same way from R2, 13% of the latter passing into the mother liquors.

(2) *Wallerstein material.* Wallerstein tyrothricin (of the batch previously studied by Gordon *et al.* (1943 *b*, *d*)) was washed with B.P. ether to remove fatty material, and worked up for gramicidin according to Hotchkiss & Dubos (1941). The first crystalline product (W1) and that obtained after a single recrystallization as above (W2) have been studied.

Comparison of gramicidin specimens

The results of this comparison are shown in Table 2. Material was dried to constant weight *in vacuo* at room temperature. Optical rotation was determined in 94% alcohol ($c = 1·5$). N was determined in duplicate by the Kjeldahl procedure after treatment with red P and HI as previously. The amino-acid contents are based on the N figures thus obtained.

For the *amino-acid analyses* the previous procedure (Gordon *et al.* 1943*b*) was followed with 9–10 mg. samples, except that: (1) hydrolysis was effected in sealed evacuated tubes with 0·8 ml. glacial acetic acid and 3·0 ml. 5N-HCl for 48 hr. at 100°; (2) the leucine fraction obtained from the *n*-propanol-cyclohexane chromatogram was not subjected to refractionation but titrated directly.

The results with a control mixture of five acetamino-acids, hydrolyzed and analyzed in exactly the same way as the gramicidin specimens, are given in Table 1.

Table 1. *Analysis of control mixture of acetamino-acids simulating gramicidin*

Compound	Taken for analysis (mg.)	Recovery (%)
Acetyl-*d*-leucine	3·75	100·5, 103
Acetyl-*l*-tryptophan	4·25	85, 98
Acetyl-*dl*-valine	2·15	103, 108
Acetyl-*dl*-alanine	1·55	97, 100 (corrected)
Acetylglycine	0·84	89, 122 (corrected)

In Table 2, which shows the analytical figures for various gramicidin specimens, the valine and tryptophan figures are corrected, 90% recovery of tryptophan and 105% recovery of valine, as found from Table 1, being assumed. (Control experiments showed that on the 3% butanol-chloroform columns with silica gel batch γ, about 5% of acetyltryptophan 'tails' into the acetylvaline fraction (Gordon *et al.* 1944).) Glycine and alanine figures are given corrected for selective extraction losses (see above).

Hydroxyamino and NH_3 determinations were done according to Gordon *et al.* (1943*c*). The hydrolysate of 5 mg. of material was evaporated in the outer chamber of a Conway dish (whose inner and outer rims had been smeared with vaseline) in a H_2SO_4-soda lime vacuum desiccator. Water and reagents were added and the determination was carried out as previously. Control experiments with $(NH_4)_2SO_4$ and *dl*-serine again showed 94% recovery, and the results have been corrected accordingly. The hydrolysate of the control mixture shown in Table 1 under these conditions yielded 0·07% of its N as NH_3 and 0·2% as hydroxyamino-N.

The appearances of all specimens during hydrolysis and analysis were indistinguishable. At 100° the flocculated material dissolved more slowly than previously in the hydrolysis mixture. After the first 24 hr. of hydrolysis,

Table 2. *Comparison of gramicidin specimens*

Specimen	m.p.	$(\alpha)_D$ at temp.		N (%)	As % of total N of gramicidin: Leucine N	Trypto-phan N	Valine N	Alanine N	Glycine N	Hydroxy-amino N	NH_3 N
R1	224–6°	+ 9·4°	21°	14·1	23·4	36·0	14·0	11·1	6·6	—	—
R2	—	+ 9·9°	17·5°	14·1	23·3	38·8	15·9	10·7	7·9	—	—
R3	225°	+11·9°	19·5°	14·1	23·5	38·4	13·9	10·1	7·4	4·3	0·3
W1	—	+ 8·5°	18°	14·0	25·7	39·1	14·3	10·6	8·4	—	—
W2	—	+ 8·0°	18°	13·9	25·2	41·1	15·7	11·7	7·8	4·6	0·2

there remained undissolved a drop of floating oil, which did not diminish in amount on further heating. No such oil was apparent with hydrolysates of the control mixture. It may have been due to contamination by difficultly removable fatty material, which may also be responsible for the low N content of the gramicidin specimens. The fact that the leucine figures are higher than formerly found is partly due to non-removal of the previously noted anomalous bands by a butanol-chloroform fractionation. These may be formed by the acetyl derivatives of difficultly hydrolyzable peptides.

Partition chromatography of free peptides on filter-paper strips

The band-rate data in Table 3 for the peptides studied with *n*-butanol-H_2O are on the R_F scale (Consden *et al.* 1944) and should be compared with the data of those authors for free amino-acids. The bands obtained were similar in width to those with free amino-acids. More vigorous heating than with free amino-acids was required for obtaining a colour with ninhydrin. Colours ranged from dull pink to bluish purple. These experiments simultaneously provided a check on the homogeneity of the peptide preparations studied.

Table 3. *Band-rates of peptides on filter-paper strips developed with* n-*butanol water*

Compound	Band-rate (R_F)
dl-Leucyl-*dl*-leucine 'A' *l*-Leucyl-*l*-tryptophan	0·8
dl-Leucylglycine Glycyl-*dl*-leucine	0·25
d-Leucylglycylglycine	0·13
dl-Valylglycine	0·08
Glycylglycine	0·03

Preparation of starch for partition chromatography

Raw potato starch, as obtained from Messrs Gordon Slater Ltd., Manchester, contained a significant quantity of material extractable by water, and gave a pale pink colour on heating with ninhydrin. This disappeared after washing by decantation six times with 10 parts by weight of distilled water. Air-dry starch (5 g.), which had been washed in this way, was extracted by keeping at room temperature for 24 hr. with 20 ml. *n*-butanol saturated with water. The extract yielded 0·4 mg. of non-volatile matter. The extracted starch was resuspended in fresh wet *n*-butanol and made into a chromatogram. After 12 ml. of this solvent had passed through the chromatogram, the next 12 ml. of effluent were collected, and yielded on evaporation 0·15 mg. of non-volatile matter. For subsequent work air-dry starch, which had been washed as above with distilled water and dried at 37° to a free-running powder, was made into chromatograms with wet *n*-butanol, and a column's length of solvent was allowed to flow through the chromatogram under gravity before applying the material for analysis. In this way contamination of the effluent by the starch was reduced sufficiently for the present work.

Since chromatograms of this kind ran more slowly than was wished, the largest granules (15% by weight of the original starch) were isolated by sedimentation and made into chromatograms. These flowed about twice as fast as those made with unfractionated starch, but corresponding amino-acid bands on them appeared nearly twice as broad. Unfractionated starch was therefore employed for all the work described here.

Behaviour of free amino-acids on starch chromatograms developed with n-*butanol*

Glycine, alanine, valine, leucine and tryptophan ran as bands on starch chromatograms prepared as above. The bands were comparable in width and mutual resolution with those obtained by the paper-strip method.

A quantitative study was made on a starch chromatogram in a tube having internal diameter 1 cm. Half milligram quantities of *dl*-alanine, *dl*-valine and *dl*-leucine were applied in a minimum of wet *n*-butanol, and the chromatogram was developed with the same solvent. When the solvent level in the chromatogram tube had fallen by a total of 9·8 cm. a 0·5% (w/v) solution of ninhydrin in B.P. ether was substituted for solvent above the column. The centres of the resulting blue bands were measured x cm. from the top of the column. To calculate R values for the bands (Martin & Synge, 1941*b*) it was assumed that the band was immobilized at the moment when reached by the ether front passing down through the column. Since the solvent in the column moved 2·3 times as fast as in the tube above the column,

$$R = \frac{x}{9{\cdot}8 + (x/2{\cdot}3)}.$$

From the R values, the partition coefficient was calculated by equation (2) of Martin & Synge (1941*b*), it being assumed that the starch grains held the same volume of aqueous phase as of water when saturated in an atmosphere having relative humidity of 1. Starch saturated in this way (5·73 g.) made a column of length 9·0 cm. The water content of the starch was found to be 38% by weight on the anhydrous starch present. The specific gravity of anhydrous starch was taken to be 1·65. The results of these calculations are compared in Table 4 with the direct determinations of α by England & Cohn (1935). The α values ascribed to these authors are those corresponding to the lowest concentration of each substance studied by them.

Table 4. *Partition coefficients of amino-acids between* n-*butanol water phases determined from band-rates on starch chromatograms and directly*

Amino-acid	Movement of band (x cm.)	R	α (from band-rate)	α (England & Cohn, 1935)
dl-Alanine	1·0	0·10	44·0	42·2
dl-Valine	3·2	0·29	13·7	13·9
dl-Leucine	7·0	0·55	6·4	5·5

Quantitative experiments on recovery of amino-acids from starch chromatograms have not yet been carried out.

Chromatographic fractionation of gramicidin partial hydrolysate

Van Slyke amino-N determinations (4 min. reaction time) on various gramicidin specimens hydrolyzed for 10 days as below gave 39, 41 and 45 % of the N as amino-N.

252 mg. gramicidin *R*3 were dissolved in 12·5 ml. glacial acetic acid, and 12·5 ml. 10 N-HCl were added to the solution. This caused no precipitation. The solution was kept in a sealed evacuated vessel at 37° for 10 days, during which time a slight fluffy precipitate formed and the solution became bluish purple. At the end of this time the mixture was evaporated to dryness *in vacuo* and kept for a further ½ hr. at 45° while evacuating with a water-pump. The gummy residue was entirely soluble in wet *n*-butanol. It was transferred quantitatively in 5 ml. of this solvent (followed by three washings of 5 ml.) to a chromatogram made from 150 g. air-dried starch in a 5·1 cm. tube.

The chromatogram was developed with fresh solvent, and in the course of 48 hr. 40 serially numbered fractions were collected in addition to a preliminary fraction (P) which preceded the fastest-moving material. In the early stages, striking red, green and bluish white fluorescent bands were observed on ultra-violet irradiation of the column. The positions of these bands are shown in Fig. 1. The red fluorescent band was associated with a visible red pigment; behind this band the column appeared quite colourless in ordinary light. The effluent fractions were stored at 0° pending investigation.

A drop of effluent from each fraction was placed as a spot on filter-paper. The paper was sprayed with ninhydrin and heated. The relative intensities of the resulting spots, as judged roughly by eye, are illustrated in Fig. 1. Those corresponding to free amino-acids (noted in Fig. 1) appeared more rapidly than the others.

Tryptophan determinations were done colorimetrically on samples of 1/200 of the volume of each fraction. The butanol was evaporated in a vacuum desiccator containing H_2SO_4; 2·5 ml. glacial acetic acid and 2·5 ml. 0·1 % (w/v) solution of *p*-dimethylaminobenzaldehyde in 10 N-HCl were added to this residue, and the resulting solution was stored in the dark for 24 hr. at 37° followed by 24 hr. at room temperature. The resulting blue colour was compared with that of a series of standards of *l*-leucyl-*l*-tryptophan (containing from 0·2 to 12·2 μg. of tryptophan N) which had been treated in the same way. Control experiments had shown that the tryptophan in gramicidin and in leucyltryptophan yielded the same amount of colour under these conditions, whereas that in acetyltryptophan yielded about 20 % less colour. The colour with leucyltryptophan, acetyltryptophan and the partial hydrolysate fractions began to be obvious immediately the reagents were added, whereas with intact gramicidin it appeared rather slowly. The results of these determinations are shown in Fig. 1.

Too much significance should not be attached to the absolute values, in view of the differences in final colour given by different tryptophan compounds. Summation of the values for all the fractions gives a recovery of about 50 % of the tryptophan originally present.

Amino-acid composition of slower-moving bands

Samples (1/200 of the volume) of each of the tryptophan-free fractions nos. 18–40 inclusive were evaporated to dryness in 'Hysil' test-tubes (Baird and Tatlock, Ltd.) in a vacuum desiccator containing H_2SO_4. The residues were dissolved in 0·12 ml. 6 N-HCl; the tubes were evacuated, sealed and heated overnight at 115°. After the tubes were opened, the acid was removed in a vacuum desiccator containing soda-lime, and a high proportion of the residue in each tube was transferred to a filter-paper strip in distilled water. The strips were developed with *n*-butanol-H_2O, and on colouring with ninhydrin gave the data embodied in Fig. 2, which records relative intensities of amino-acid bands judged by eye. The experiment was repeated with development of the strips with phenol-H_2O, which permitted easier distinction between glycine and alanine, and confirmed the previous result.

On the basis of this information, the fractions were placed in groups *A*, *B*, *C*, *D* and *E* as shown in Fig. 2. Samples (1/5 volume) from all the fractions of each group were bulked, and evaporated to dryness below 40° (with a Hyvac pump and an acetone-solid CO_2 bath for condensation). The residues were hydrolyzed in 6 N-HCl at 100° for 24 hr. under a reflux condenser, and analyzed quantitatively on silica gel chromatograms after acetylation, as described above. For each analysis a single 5 % *n*-propanol-cyclohexane column was employed, the

Table 5. *Amino-acid composition of fractions obtained from gramicidin partial hydrolysate*

Fraction-group	As % of total N of original gramicidin			
	Leucine N	Valine N	Alanine N	Glycine N
A	← 0·22* →		2·50	0·17*
B	← 0·14* →		0·15	0·19
C	0·07*	2·46	1·32	2·49
D	← 0·22* →		← 0·40* →	
E	0·49	0·67	0·23	0·53

Figures marked with asterisks were obtained from 'blind' fractions, no corresponding acid band being visible on the chromatograms.

The small quantities of 'background' material in the asterisked fractions may account for some of the material giving fluorescence and colour with ninhydrin (cf. Fig. 1). These substances were not detected in the qualitative amino-acid analysis shown in Fig. 2.

valine, alanine and glycine fractions being run out with 30% *n*-propanol-cyclohexane. The results of these analyses are given in Table 5.

Further investigation of fraction-group A

A further sample (1/5 volume) of this material was evaporated to dryness *in vacuo* below 60°, yielding a crystalline residue. Dissolved in water, the material yielded a slight precipitate on addition of 1 drop of 0·1 M-$AgNO_3$, but required only 0·27 ml. 0·01 N-$Ba(OH)_2$ to render the solution neutral to bromthymol blue, suggesting that mainly free alanine, and not its hydrochloride, was present. The resulting solution was evaporated, acetylated, extracted and analyzed chromatographically, employing 17% *n*-butanol-chloroform as developing solvent. A strong band, having the characteristic rate for acetylalanine, was collected, and was found on titration to account for 90% (corrected) of the alanine content of the fraction (Table 5). The band was followed by a much fainter band, having $R = 0{\cdot}1$, which was not further studied. The material of the main band, after titration, was hydrolyzed in 6 N-HCl for 24 hr. at 100°, and subjected to quantitative amino-acid analysis with a single *n*-butanol-chloroform chromatogram. Only alanine was observed and this amounted to 86% (corrected) of a molecule per —COOH group present in the material analyzed.

Further investigation of fraction-group C

Control experiments with synthetic peptides. 3·66 mg. glycyl-*dl*-valine were hydrolyzed and subjected to amino-acid analysis in exactly the same way as in the analyses of Table 5. It yielded 98% of the theoretical valine and 90% (corrected) of the theoretical glycine. No other acid bands were noted in the chromatogram.

3·62 mg. *dl*-valylglycine treated in the same way gave exactly the same appearance on the chromatogram as fraction-group C had done, yielding 63·5% of an equivalent of acetylvaline and 57% (corrected) of an equivalent of acetylglycine per molecule. The band corresponding in rate to acetylalanine (i.e. acetyl-*dl*-valylglycine) yielded 31% (corrected by alanine factor) of an equivalent per molecule.

Optical rotation. All that remained of fraction-group C (77% of the volume) was evaporated to dryness *in vacuo* below 40°, yielding an amorphous product, soluble in ethanol and precipitated from this solution as an oil on addition of ether. On keeping at 0° the oil deposited a few spherulitic crystal growths, which did not increase in size on further keeping. The 23 mg. of amorphous product initially obtained were freed from traces of fatty material by precipitation of a very strong ethanolic solution with excess of ether. 3 mg. were lost in this way, and the product was entirely soluble in water. It gave $(\alpha)_D^{18\cdot5°}$, +58° (water, $c = 1{\cdot}25$). The rotation is calculated for *valylglycine*, the amount being that estimated from the valine content of the fraction, determined as valine and 'alanine' in Table 5. The highest activity recorded for *l*-valylglycine by Fischer & Scheibler (1908*a*), is $(\alpha)_D^{20°}$, +93·6° (water, $c = 10$); $(\alpha)_D^{20°}$, +39·4° (10% HCl, $c = 2{\cdot}8$).

Electrometric titration. This was done with 0·1 N-HCl and NaOH on 12–13 mg. samples of an initial volume of 5 ml. and at 22°. A Cambridge pH meter with glass electrode was used. The solutions were first taken to pH 2·2 with HCl, then to about pH 10 with NaOH, and then further HCl was added to make the total addition equivalent to the NaOH used. Both the curves of descending pH agreed closely with the curves of ascending pH, and the final pH in each case lay within 0·1 unit of the initial pH. In view of the coincidence of the curves and for clarity of the diagram, only the curves of ascending pH are given (Fig. 3). *dl*-Valylglycine and its diketopiperazine (*dl*-3-*iso*propylpiperazine-2:5-dione) were compared with the natural product recovered from the determination of optical rotation. The *dl*-valylglycine and its anhydride gave initial pH's of 5·7 and 6·0 respectively, whereas the natural product had an initial pH 2·8 and its solution gave a strong precipitation with $AgNO_3$, demonstrating that it had emerged from the chromatogram largely as the hydrochloride.

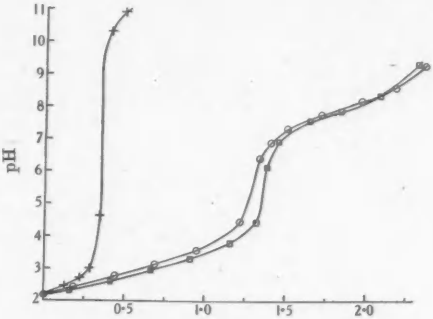

Fig. 3. Comparison by electrometric titration of materia in fraction-group C with synthetic materials. +—+ *dl*-3-*iso*Propylpiperazine-2:5-dione. □—□ *dl*-Valylglycine. o—o Natural product.

In Fig. 3 the pH is plotted against equivalents of NaOH per unit of substance embodying one valine residue. With the natural product the same valine figure is employed as in the optical measurement above.

Acetyl derivative. The solution from the electrometric experiment was evaporated to dryness *in vacuo* below 40°, acetylated and extracted as for a quantitative amino-acid analysis. With this material on a 17% *n*-butanol-chloroform chromatogram of suitably enlarged diameter, a single acid band was observed, having the same rate as acetylalanine. The material from this band was divided in solution into three equal portions—I, II and III.

Solution I was evaporated and stored for reference. The residue crystallized readily.

Solution II was evaporated and weighed after drying to constant weight in a vacuum desiccator over H_2SO_4 and soda-lime. The material was then dissolved in water and titrated to bromthymol blue with 0·01 N-$Ba(OH_2)$. It was thus found to have acid equivalent weight 220. *Acetyl-l-valylglycine*, $C_9H_{16}O_4N_2$, requires acid equivalent weight 216 (synthetic preparation of acetyl-*dl*-valylglycine, Steiger, 1934*a*). The yield of acetylvalylglycine after acetylation

and extraction was 80% of the theoretical, based on the estimated valine content of the original material.

The material from this titration was hydrolyzed in 6N-HCl in a sealed evacuated tube for 36 hr. at 115°. On quantitative amino-acid analysis, employing a *n*-propanol-cyclohexane column, it yielded 95% of a molecule of valine and 88% (corrected) of a molecule of glycine per —COOH group initially present. No other acid bands were observed on the chromatogram. A small sample was employed for paper-strip chromatograms (*n*-butanol-H_2O), in which the hydrolysate was compared by the 'mixed chromatogram' technique with a hydrolysate of synthetic *dl*-valylglycine and with a made-up mixture of *dl*-valine and glycine applied as the hydrochlorides. No divergences were detected in this way.

Optical activity of valine resulting from acid hydrolysis of dipeptide

Solution III (see previous paragraph) was evaporated, and the residue treated with 6N-HCl in a sealed evacuated tube at 115° for 36 hr. The hydrolysate was acetylated and extracted in the usual way. To the extract was added the acetylvaline obtained in the analysis of the previous paragraph, and of fraction-group C (Table 5). It was liberated from its Ba salt by addition of the calculated quantity of HCl.

The combined material was then subjected to chromatographic purification on a 3% *n*-butanol-chloroform silica gel column. The acetylvaline fraction thus obtained was hydrolyzed overnight in 6N-HCl at 100° and evaporated to dryness. The optical activity of the residue in 6N-HCl was $(\alpha)_D^{18\cdot5°}$, $+22°\pm3°$ ($c=0\cdot9$) (calc. for *valine*, the amount present being known from the above analyses). This is a high activity in view of the numerous manipulations that might have caused racemization.

Optical activity of valine resulting from hydrolysis of gramicidin

A. 34·6 mg. gramicidin W2 were hydrolyzed in a mixture of 1·5 ml. glacial acetic acid and 1·5 ml. 10N-HCl in a sealed evacuated tube for 10 days at 37°. The mixture was then kept at 115° for 36 hr. Acetylvaline was then prepared from the hydrolysate by exactly the procedure used in quantitative amino-acid analyses of gramicidin (above), using chromatogram tubes of enlarged diameter. The product was hydrolyzed in 6N-HCl in a sealed evacuated tube at 100° overnight; this hydrolysate was evaporated to dryness. The optical activity of the residue in 6N-HCl was $(\alpha)_D^{20°}$, $-10°\pm3°$ ($c=1\cdot0$) (calc. for *valine* with the figure of Gordon *et al.* (1943*b*) for the valine content of gramicidin, 100% recovery being assumed).

B. 34·1 mg. gramicidin W2 were worked up in exactly the same way, except that it was hydrolyzed immediately at 115°, omitting the treatment at 37°. For the valine fraction, $(\alpha)_D^{20°}$ was $0°\pm3°$ (6N-HCl, $c=1\cdot0$), calculated on the same basis.

SUMMARY

1. Specimens of gramicidin from two different sources have been compared in respect of a number of properties and further information has been obtained about the ultimate hydrolysis products.

2. Preliminary data are provided on the use of raw potato starch as a medium for partition chromatography of free amino-acids and peptides.

3. Some results are given of the analysis of a partial hydrolysate of gramicidin by this method. From one of the fractions studied the peptide *l*-valylglycine has been obtained in a yield embodying more than half of the glycine of gramicidin.

4. The optical form of the valine residues of gramicidin is discussed in the light of new evidence. It is probable that *d*-valine residues will be discovered to be structural components of gramicidin.

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The Esterases of Skin

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The enzyme content of skin has received little attention; yet the risk of exposure of this tissue to toxic agents, a risk that is increasing with modern industrial processes, and the attendant possibility of damage to essential skin enzymes, makes any addition to knowledge in this direction desirable. Accordingly, when it was found that acetylcholine was rapidly hydrolyzed by rat-skin slices, it was decided to examine this activity in greater detail.

The presence of a cholinesterase in skin does not appear to have been reported previously, though from the work of Langley (1923), Lewis & Grant (1924), Dale (1933), Dale & Feldberg (1934) and others on the innervation and vascular reactions of the skin, it was to be expected that certain of the structures in skin would be associated with cholinesterase activity.

Since the work of Vahlquist (1935) there has been some doubt as to whether all cholinesterase activity is due to a specific enzyme. Richter & Croft (1942), Mendel & Mundell (1943), Mendel, Mundell & Rudney (1943) and Mendel & Rudney (1943) agree that the cholinesterase of red blood cells is specific, while that of the serum of certain species is non-specific. Mendel & Rudney (1943) also obtained a non-specific cholinesterase from dog pancreas, and have suggested the name pseudo-cholinesterase for this type of enzyme. Further, Alles & Hawes (1940), Glick (1941) and Richter & Croft (1942) have each described differences in the specificity and other properties of cholinesterase preparations from different sources, and the last-named workers have suggested that the cholinesterases should more properly be regarded as a group of related enzymes.

The first step in the further investigation of the cholinesterase activity of skin was therefore to decide whether or not it is due to a specific enzyme. In our opinion the evidence now to be presented supports the view that it is a true specific cholinesterase.

METHODS

Estimation of esterase activity

Esterase activity was determined manometrically with Ammon's (1933) adaptation of the Warburg technique, in which the acid produced by enzymic hydrolysis is measured by CO_2 evolution from bicarbonate buffer at pH 7·4. Skin slices were suspended in 0·025 M-$NaHCO_3$ (total volume after additions, 3 ml.), the gas space being filled with 95% N_2+5% CO_2. All measurements were made in duplicate at 38°, the majority lasting for a total period of 1 hr., intermediate readings being taken after 15 or 30 min.

Non-enzymic hydrolysis, which was in all cases small, was controlled by including an additional pair of bottles containing no substrate. A correction was also made in each experiment for the acid production of the tissue in the absence of added substrate, which again was always small.

Esterase activity is expressed as μl. CO_2/g. tissue (wet wt.)/hr., except where otherwise stated.

Substrates

Three substrates, acetylcholine chloride (ACh), methyl butyrate (MB) and tributyrin (TB), were used; the first two were added as solutions in 0·025 M-$NaHCO_3$ (final concentration 0·015 and 0·03 M respectively), while the third was pipetted directly into the bottles, 0·05 ml. being added unless otherwise stated. Under our conditions these concentrations were found to give a constant rate of gas evolution over two successive 30 min. periods.

Source of tissue

Experiments were carried out on three kinds of skin.

Adult rat skin. The fur was clipped from an area of 30–40 sq. cm. of the belly, flanks and chest of normal white rats (weighing not more than 250 g.) under light ether anaesthesia. 1–2 hr. after the end of the anaesthetic the rat was decapitated, the skin dissected away, scraped free from underlying adipose and connective tissue, and sliced with a razor. 150–200 mg. were added to each bottle. Histological examination of the skin prepared in this way showed that subcutaneous tissue had been effectively removed.

Young rat skin (hairless). After decapitation of the rat the skin was removed from the entire trunk, scraped and sliced as before. 50–100 mg. were added to each bottle.

Human skin. Two experiments were carried out with samples of normal skin from two human subjects; in each case the skin was obtained from the breast immediately after surgical removal.

RESULTS

Cholinesterase activity of skin. Table 1 shows the cholinesterase activity of rat-skin slices found in the original experiments. In these early experiments the activity was measured over only 30 min. It will be seen that the level of activity shows but slight variations among the different animals in the weight-range studied. The values, moreover, agree closely with those found for adult rats in the present work, which are presented in Table 2. This table also includes data from young rat skin and human skin.

Table 1. *Cholinesterase activity of rat-skin slices*

Wt. of rat (g.)	Enzyme activity (μl. CO_2/g./hr.)
33	1356
56	1156
58	1124
59	1220
60	1180
62	1066
65	1114
74	1038
84	1046

The former differs little from the adult skin; human skin is somewhat less active.

Hydrolysis of tributyrin and methyl butyrate. The first step in investigating the specificity of this activity against acetylcholine was to determine whether skin possesses esterase activity towards other esters, particularly simple aliphatic esters. In conformity with the earlier work on the specificity of the cholinesterases of blood (Stedman, Stedman & Easson, 1932; Stedman, Stedman & White, 1933), tributyrin and methyl butyrate were selected as substrates.

Table 2. *Hydrolysis of acetylcholine, tributyrin and methyl butyrate by skin slices*

(Enzyme activity expressed as μl. CO_2/g./hr.)

Acetylcholine	Tributyrin	Methyl butyrate
	Adult rats	
1079	4153	—
1492	3093	—
933	4427	—
1186	3938	—
1466	3706	—
1263	4922	—
1140	3863	—
1033	4687	—
1004	3001	—
1298	5051	—
1680	4952	8258
	Young rats	
1480	1777	1774
1377	2293	2597
1499	1626	5172
1871	2725	1529
1554	3227	1015
1182	3902	1112
1497	2170	4463
1445	2409	2538
	Human	
406	949	—
565	1142	—

Table 2 shows that tributyrin is split even more rapidly than acetylcholine by both adult and young rat skin as well as by human skin. In the case of methyl butyrate the hydrolysis is still more rapid, so that observations with this substrate were restricted to young rat skin, in which the hydrolysis proceeds at a more conveniently measurable rate.

Further, it will be seen from Table 2 that, by contrast with the relatively constant values obtained with acetylcholine, the hydrolysis rates of tributyrin and methyl butyrate by young rat skin showed considerable variation in the different experiments. This variation was seen to be related to the age of the animal, a point that will be dealt with in a later section.

Sensitivity to eserine. The first test to determine the specificity of the skin cholinesterase activity was a comparison of the sensitivity of the different esterase activities to eserine. Varying amounts of eserine (British Drug Houses Ltd., physostigmine sulphate) were dissolved in 0·025 M-$NaHCO_3$, and added to the bottles immediately after the substrate.

Table 3. *Effect of eserine on hydrolysis of acetylcholine* (ACh), *tributyrin* (TB) *and methyl butyrate* (MB) *by skin slices*

(Enzyme activity expressed as μl. CO_2/g./hr.)

Concentration of eserine ($\times 10^{-5}$ M)	ACh	ACh + eserine	TB	TB + eserine	Inhibition ACh (%)	Inhibition TB (%)
			Adult rats			
0·5	1263	297	3093	2584	77	16
	1235	396	3001	2509	68	16
1·0	1079	0	—	—	100	—
	1492	4	—	—	100	—
	933	0	—	—	100	—
	1186	52	—	—	96	—
	1140	98	4427	3700	92	16
	1147	0	5051	4898	100	3
5·0	1033	13	3706	3089	99	17
	1128	0	4952	4587	100	7
			Young rats			
0·5	1871	439	2725	2407	77	12
1·0	1480	121	1777	1670	92	6
	1377	9	2293	1629	99	29
5·0	1554	4	3227	2563	100	21
			Human			
1·0	406	0	949	756	100	20
	565	0	1142	1013	100	11

Concentration of eserine ($\times 10^{-5}$ M)	ACh	ACh + eserine	MB	MB + eserine	Inhibition ACh (%)	Inhibition MB (%)
			Young rats			
1·0	1182	162	1774	1789	87	0
	1497	47	2597	2159	97	17

Table 3 shows the effect of 0·5, 1·0 and 5·0 $\times 10^{-5}$ M eserine sulphate on the rates of hydrolysis of acetylcholine and tributyrin by adult rat skin. The cholinesterase activity is seen to be strongly inhibited at the lowest concentration, and completely at the other two, while the tributyrin activity is only slightly affected at all the concentrations used. This marked difference is also observed in young

rat skin and human skin. Two experiments with young rat skin and methyl butyrate included in Table 3 show the same insensitivity to eserine. In all the experiments quoted in Table 3 readings were taken over two successive 30 min. periods; no significant change was noted in the eserine inhibition of any of the substrates in the two periods, and the small and variable inhibition of tributyrin and methyl butyrate hydrolysis was not significantly increased by a tenfold increase in the concentration of eserine.

Experiments with mixed substrates. The second criterion of specificity was obtained from summation experiments in which the CO_2 evolution of bottles containing single substrates was contrasted with that of bottles containing two substrates.

If two separate esterases, specific for the two substrates, are present, the CO_2 evolution of the bottles containing the mixed substrates should equal the sum of the CO_2 evolutions of the bottles containing the single substrates, provided the substrate concentrations are adequate to saturate the enzyme systems present and the presence of the one substrate does not inhibit the hydrolysis of the other. If, on the other hand, one enzyme is responsible for the hydrolysis of the two substrates, no summation could take place at these substrate concentrations owing to saturation of the enzyme. The results presented in Table 4 show that the former type of behaviour is obtained with acetylcholine if either methyl butyrate or tributyrin is the second substrate.

was used (2 μl. pipetted into the bottles by means of a micropipette), a preliminary experiment having shown that this would give a CO_2 evolution approximately equal to that of the acetylcholine. A rough determination of the Michaelis constants for tributyrin and acetylcholine showed that they were approximately equal (0·003 M), so that an objection to Exp. 904 is that, if a single enzyme is involved, it would not be saturated by the tributyrin and so might bring about the hydrolysis of both substrates. In the next three experiments in Table 4, 0·02 M tributyrin was used (0·02 ml. pipetted), this concentration lying on the 'plateau' of the substrate concentration-activity curve. In Exp. 920 the skin from an 8 g. rat was used; with animals of this weight the tributyrin activity is so much lower that rates of hydrolysis comparable with those of acetylcholine are obtained; in this experiment 0·05 ml. of tributyrin was added. It will also be seen from Table 4 that the CO_2 evolution remains reasonably constant over the two 30 min. periods studied.

The rate of hydrolysis of methyl butyrate by adult rat skin is so much greater than that of acetylcholine that no attempt was made to carry out summation experiments with this tissue. Satisfactory experiments were, however, carried out with very young rats (6 and 7 g.); these are also given in Table 4.

The results of all these experiments clearly indicate summation with acetylcholine and either tributyrin or methyl butyrate, and provide further evidence that the cholinesterase is distinct from

Table 4. *Effect of mixed substrates on activity of skin*

(Enzyme activity expressed as μl. CO_2/g./30 min.; a = 0–30 min., b = 30–60 min. period of experiment.)

	Acetylcholine		Tributyrin		Acetylcholine + tributyrin		Comparison of found and calc. CO_2 evolution from mixed substrates (μl. CO_2/g./hr.)	
Exp.	(a)	(b)	(a)	(b)	(a)	(b)	Found	Calc.
904	513	—	693	—	1128	—	—	—
910	415	519	2125	2219	2754	2482	5236	5278
912	549	561	1247	1285	1528	1480	3008	3642
914	622	585	1561	1741	2354	2396	4750	4509
920	619	880	841	785	1425	1665	3090	3125

	Acetylcholine		Methyl butyrate		Methyl butyrate + acetylcholine		Comparison of found and calc. CO_2 evolution from mixed substrates (μl. CO_2/g./hr.)	
Exp.	(a)	(b)	(a)	(b)	(a)	(b)	Found	Calc.
932	529	755	752	777	1298	1476	2774	2813
933	656	708	499	516	1287	1123	2410	2379

In the case of the tributyrin experiments the much more rapid hydrolysis of this substrate by adult rat skin presented a difficulty. In the first summation experiment (Exp. 904) 0·002 M tributyrin the esterase (or esterases) splitting these aliphatic esters.

By contrast with the complete summation of acetylcholine with either tributyrin or methyl buty-

rate hydrolysis, three experiments with mixed tributyrin and methyl butyrate yielded inconclusive results, there being some evidence of a small degree of summation. On the other hand, mixed methyl and ethyl butyrate, substrates which might confidently be expected to be hydrolyzed by the same enzyme, showed no evidence of summation (Table 5), thereby confirming the interpretation put on the positive summation with acetylcholine as one of the substrates.

Table 5. *Hydrolysis of mixed methyl and ethyl butyrate by rat-skin slices*

(Enzyme activity expressed as μl. CO_2/g./30 min.)

	Period (min.)	
	0–30	30–60
Methyl butyrate	1074	1045
Ethyl butyrate*	1157	1168
Methyl butyrate + ethyl butyrate	1078	1055

* Ethyl butyrate, 0·01 ml./3 ml.

Variation of esterase activity with age. In the course of the foregoing experiments it became clear that the different esterase activities of rat skin showed a marked divergence in their respective variations according to the age of the animal. This is illustrated in Table 6. The rates of hydrolysis of

Table 6. *Variations of skin esterase activity with age*

(Enzyme activity expressed as μl. CO_2/g./hr. Numbers in brackets denote the number of experiments with rats of any one age group.)

Wt. of rat (g.)	Acetylcholine	Tributyrin	Methyl butyrate
3–6	1395 (3)	2293	1015
7–8	1545 (3)	2270 (4)	1449 (3)
10	1554	3227	2162 (3)
13–15	1345 (2)	—	2186 (2)
20	—	3885 (2)	4818 (2)
54	1680	—	8258
80–100	1168 (3)	—	—
145–200	1222 (6)	4335 (5)	—
225–230	—	4805 (2)	—

acetylcholine and methyl butyrate are approximately equal in very young rats, while that of tributyrin is slightly higher; but whereas the cholinesterase activity does not significantly change with increasing weight, the tributyrin activity increases somewhat, and the methyl butyrate activity markedly. This may be regarded as still further evidence that the enzyme hydrolyzing acetylcholine is distinct from that affecting these other esters. Further, the markedly greater increase in activity towards methyl butyrate with increasing age over that towards tributyrin, together with the slight evidence of partial summation with these two substrates, suggests the possibility that two distinct enzymes may be attacking these two substances.

DISCUSSION

The numerous studies of the cholinesterase activity of various animal tissues (Plattner & Hintner, 1930; Gilman, Carlson & Goodman, 1939) do not appear to include skin. It was thought desirable to investigate the cholinesterase activity of skin, particularly as certain pathological conditions of skin may be associated with abnormal functioning of cholinergic nerves. Grant, Pearson & Comeau (1936), for example, have investigated a series of cases of urticaria of nervous origin, and have concluded that the condition is brought about by the release of acetylcholine in the skin as a result of stimulation of cholinergic nerve fibres. Alexander, Elliott & Kirchner (1940) have stated that eserine, introduced electrophoretically into the skin, is a powerful urticariogenic substance even in dilutions of 1:10,000 and occasionally 1:100,000.

The results described in this paper show that both rat and human skin possess an active cholinesterase. That this is a specific enzyme has been shown by comparing the sensitivities to eserine of the enzymic hydrolysis of acetylcholine, tributyrin and methyl butyrate, both of which latter substrates are also rapidly hydrolyzed by rat skin. Confirmation of this conclusion has been obtained by summation experiments with acetylcholine and either tributyrin or methyl butyrate.

It was also found that while the cholinesterase activity of skin remains approximately constant over the weight range of animals studied, the rates of hydrolysis of tributyrin and methyl butyrate increase with increasing age. This increase is in striking contrast with the reported changes in the respiration of skin, which diminishes with increasing age; Adams (1936), for example, found that the Q_{O_2} for skin from very young rats falls at 4–6 weeks to a persistently low value.

SUMMARY

1. Rat and human skin have been shown to have cholinesterase activity.

2. Rat skin also contains an esterase (or esterases) which hydrolyzes tributyrin and methyl butyrate.

3. The cholinesterase activity is very sensitive to eserine, being completely inhibited by 10^{-5}M eserine sulphate. The aliphatic esterase on the other hand is insensitive.

4. Summation experiments with mixed substrates confirm the conclusion that the cholinesterase and aliphatic esterase activities are due to different enzymes.

5. Whereas the cholinesterase activity remains approximately constant, the tributyrin activity increases somewhat, and the methyl butyrate activity increases markedly with age.

6. It is concluded that the cholinesterase of skin is a true, specific cholinesterase.

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Bacterial Tetrathionase: Adaptation without Demonstrable Cell Growth

(*A Report to the Medical Research Council*)

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In suitable conditions micro-organisms react to changes in their chemical environment by changes in enzymic activity. Two types of process may be distinguished. In the first, the new property develops slowly in successive cultures and, once developed, remains stable even in the absence of the specific substrate. In the second, the new property appears after a delay of only a few hours when the cells are brought into contact under appropriate conditions with the specific substrate, but is rapidly lost when they are subcultured in an environment not containing it (Dubos, 1940). Yudkin (1932) suggested that the term 'training' be used for the first process, and 'adaptation' for the second. 'Training' may well be brought about by natural selection. 'Adaptation' is almost certainly not, since the time for development of the new property is too short for appreciable cell division to have occurred. With the formic hydrogenlyase of *Esch. coli*, Stephenson & Stickland (1932) showed that adaptation could occur without evidence of cell division but only in an environment which was favourable for maximal growth, since they had to add full strength nutrient broth to their suspensions in order to produce adaptation. Stephenson & Yudkin (1936), however, found with the galactozymase of *Saccharomyces cerevisiae* that adaptation could occur slowly in the absence of added nitrogen source and without any significant cell division, while Hegarty (1939) found that washed suspensions of *Streptococcus lactis* could be adapted to ferment galactose in the absence of either nitrogen source or cell division. Pollock, Knox & Gell (1942) and Pollock & Knox (1943) showed that washed suspensions of *Bact. paratyphosum* B prepared from agar cultures could be adapted to reduce sodium tetrathionate rapidly to thiosulphate in accordance with the equation

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3,$$

simply by preliminary treatment for 2–3 hr. at 37° with tetrathionate and a suitable hydrogen donator. The present paper gives in detail the evidence that this adaptation occurs without detectable growth.

METHODS

Several strains of *Bact. paratyphosum* B were used; some were freshly isolated from clinical cases, others were stock laboratory cultures maintained on egg slopes. One ml. of an 18 hr. broth culture was inoculated on to the surface of bottles containing 30–40 ml. of nutrient agar prepared with

tryptic digest broth; after allowing the inoculum to run over the whole surface the bottles were incubated for 15–18 hr. at 37°. The growth was then washed off with 5–6 ml. of ¼ strength Ringer's solution (abbreviated: '¼ Ringer') per bottle, the washings were centrifuged and the deposit resuspended in 2 ml. of '¼ Ringer' solution for each bottle. A solution containing phosphate buffer (pH 7·6), phenol red, iodide-free sodium tetrathionate and mannitol was prepared and warmed to 37°. On addition of the bacterial suspension, separately warmed to 37°, the suspension mixture contained 0·2M phosphate buffer, 0·002% phenol red in ethanol (giving a final concentration of 1% ethanol in the suspension mixture), 0·02M tetrathionate, 0·02M mannitol and bacterial suspension in a dilution of 1 in 5. Reduction of tetrathionate at 37° was recorded by removal of samples at intervals during the reaction and titration with 0·005N-I_2 (Pollock & Knox, 1943). When all the tetrathionate had been reduced the remainder of the suspension mixture was centrifuged and the deposit resuspended to give about the same final concentration of bacteria in a solution of the same composition as before. Reduction of tetrathionate at 37° was again observed by the same technique.

Total and viable counts were made on the suspension mixture at the beginning and end of the adaptation process.

Total counts. A 1 mm. Helber counting chamber (depth 0·02 mm.) with a 1 mm. dark ground condenser and ×25 compensating eyepiece was used with a 16 mm. objective (Wilson, 1922). A convenient dilution for counting was 1/500 of the suspension mixture (in 1% phenol). Duplicate series of dilutions were made for all counts, and except in Exps. 11 and 12, duplicate counts were made on each dilution. At least two rows of 20 squares (i.e. 2×10^{-6} ml.) were counted in each estimation. A rough estimate of the average bacterial size was made at the time of counting. For the *viable counts*, duplicate series of dilutions were made in '¼ Ringer' and the number of colonies developing from a suitable dilution on the surface of agar plates was counted. The counts in all cases were done in duplicate and in two experiments comparison with the standard pour-plate method showed good agreement. *Dry weights* were estimated by taking 5 ml. samples, centrifuging, washing with distilled water and drying at 100–105° to constant weight. In the last two experiments nephelometric comparison of the suspension mixture at the beginning and end of the adaptation process was made by Dr Elkins, using a photo-electric nephelometer.

RESULTS

A typical experiment is illustrated in Fig. 1. Reduction of tetrathionate by a suspension of organisms from nutrient agar in the presence of mannitol proceeded at a rapidly increasing rate (curve *A*) until, after about 2 hr., the rate became linear. The same suspension (centrifuged after adaptation and resuspended in buffer-mannitol-tetrathionate solution) showed linear reduction from the start at a rate approximately equal to that of the original suspension at the end of the adaptation process (curve *A'*). This linear rate in the second half of the experiment is in fact evidence that the adaptation process was complete by the end of the first half. With unadapted suspensions the initial rate of tetrathionate reduction is too low for accurate measurement: there may or may not be a genuine lag period before any increase in rate begins. In the middle part of the curve the iodine values (indicating the amount of tetrathionate reduced to

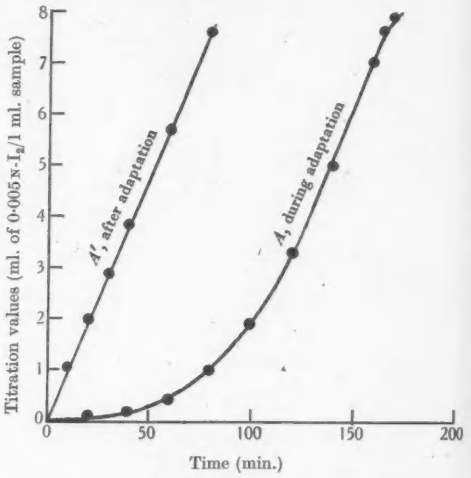

Fig. 1. Tetrathionate reduction by *Bact. paratyphosum* B during and after adaptation. Suspension mixture contained 0·02M tetrathionate, 0·2M phosphate, and 0·02M mannitol. (Complete bacterial reduction of 1 ml. of 0·02M tetrathionate to thiosulphate gives a titration value of 8 ml. of 0·005N-I_2.)

thiosulphate) increase at an approximately logarithmic rate, until the linear phase is reached when adaptation appears to be complete. Table 1 gives the results of 12 consecutive experiments designed to show whether any increase in bacterial protoplasm occurs during the adaptation process. It can be seen that there is no evidence of any increase in either the total or viable cell counts; if anything, there is a suggestion of a slight fall in the viable count during the course of the experiments. The dry weights show no significant increase, though the possibility of a slight increase in weight during adaptation cannot be dismissed. No difference in bacterial size before and after adaptation was noticed in any of the experiments. In the last two experiments duplicate 1/500 dilutions of the suspension mixtures were examined nephelometrically: the readings were identical at the beginning and the end of the adaptation process. Several strains of *Bact. paratyphosum* B were used in these experiments.

'Conway' was a stock strain which had been subcultured several times at long intervals on egg slopes; 1838 was a recently isolated strain from a case of paratyphoid B fever;

Table 1. *Reduction of sodium tetrathionate by suspensions of* Bact. paratyphosum *B*

Exp. no.	Strain	Remarks	Per ml. suspension mixture*: Beginning: Total count $\times 10^9$	Beginning: Viable count $\times 10^9$	Beginning: Dry wt. (mg.)	End: Total count $\times 10^9$	End: Viable count $\times 10^9$	End: Dry wt. (mg.)	Rate of tetrathionate reduction	Duration of exp. (min.)
1	Conway	Kept 1 day at 4°	~	~	3·3	~	~	3·2	4·8	150
2	Conway	Kept 3 weeks at 4°	19·4	16·2	3·1	17·5	13·4	3·1	1·65	180
3	Conway	—	13·3	11·4	~	12·8	9·93	~	4·4	180
4	1838	Kept 3 weeks at 4°	35·0	10·35	~	35·0	10·7	~	4·6	180
5	1838	Freshly prepared	16·0†	18·0	~	21·2	17·4	~	9·2	97
6	1838	Same suspension as 5. Kept 5 days at 4°	20·6	17·5	~	20·3	13·7	~	4·2	150
7	Murdoch 1	Kept 2 yr. on egg, used fresh	23·2	16·3	~	21·6	12·3	~	2·0	210
8	Murdoch 2	Freshly isolated, used fresh	21·0	20·5	~	20·5	16·0	~	2·0	180
9	3135	Freshly isolated, used fresh	26·15	18·9	~	24·0	14·7	~	3·2	180
10	3332	Isolated 5. viii. 43. Kept 1 day at 4°	33·7	25·6	4·5	33·6	21·65	4·6	8·0	165
11	3332	Isolated 5. viii. 43. Kept 1 day at 4°	48·75	41·1	8·6	48·6	40·0	8·72	7·0	165
12	266	Freshly isolated. Kept 1 day at 4°	24·75	19·1	4·4	24·0	15·1	4·78	7·0	165

* Total and viable counts are expressed as number of cells $\times 10^9$/ml. suspension mixture, dry weights as mg./ml. suspension mixture. Rates of tetrathionate reduction are expressed as iodine values (in terms of 0·005 N-I_2) given by 1 ml. samples of the suspension mixture. (Complete reduction of 1 ml. of 0·02 M tetrathionate gives a reading of 8 ml. of 0·005 N-I_2.) Rate of tetrathionate reduction at beginning of experiment was always low, and usually about 0·2.

† Result presumably inaccurate.

'Murdoch 1' was originally isolated (2 years previously), during the acute stage of her illness, from a woman who subsequently became a chronic carrier; 'Murdoch 2' was freshly isolated from the same patient 2 years later; 3135 was freshly isolated from a case; and 3332 and 266 were freshly isolated from two carriers remaining from two different outbreaks of paratyphoid B fever.

The interval between the preparation of a suspension and its use in an experiment was not always the same (see Exps. 5 and 6). It can be seen that the ratio between the concentration of bacterial suspension and the final rate of tetrathionate reduction varied widely in different experiments. Once fully adapted in this way these suspensions retained their power of rapid and linear reduction of tetrathionate for long periods. Repeated washing, though it slightly decreased the actual rate of tetrathionate reduction, never caused any deviation from a linear reaction. Further, these suspensions, if kept at 4° for several weeks, still gave a linear reaction though the rate slowly diminished with time of storage. The same suspensions, however, rapidly lost the newly acquired property of linear reduction on subculture and growth in a tetrathionate-free medium.

Conditions for maximal adaptation. In all the experiments of which details are given in Table 1 and Fig. 1 'adaptation' curves similar to curve *A* in Fig. 1 were obtained. This may therefore be regarded provisionally as the 'normal' or typical curve, at least for the particular conditions of these experiments. Additional experiments showed that certain alterations in the composition of the suspending fluid produced deviations in shape and velocity from the 'normal' curve. Different concen-

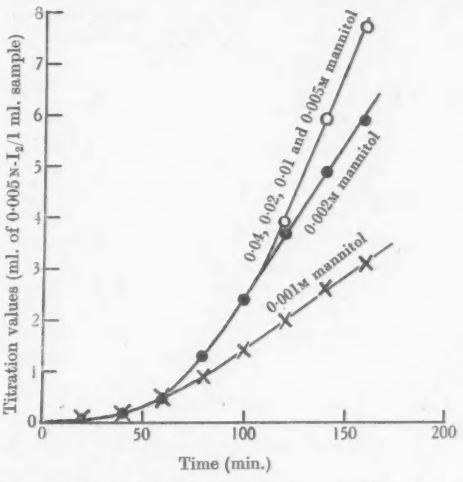

Fig. 2. Effect of different mannitol concentrations during adaptation. Suspension mixtures as in Fig. 1, but with mannitol in concentrations from 0·04 to 0·001 M.

trations of the constituents of the complete suspending fluid were tested. (1) *Phenol red* in the concentration used (0·001 %) had a definite delaying

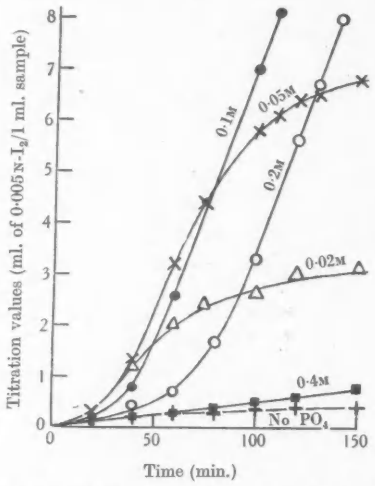

Fig. 3. Effect of different phosphate concentrations during adaptation. Suspension mixtures as in Fig. 1, but with phosphate in concentrations from 0·4 to 0·02M, and also with phosphate omitted.

effect on the reaction. This was due to the ethanol with which the indicator was made up. The final concentration of ethanol in the suspension mixture was 1 %, and 1 % ethanol alone without phenol red had exactly the same inhibitory effect. (2) *Mannitol.* Identical curves were obtained between 0·04 and 0·005M; below 0·005M the curve was atypical (Fig. 2). Mannitol was used rather than glucose, which gave even more rapid curves but from which *Bact. paratyphosum* B produces acid more rapidly, making the pH difficult to control. (3) *Phosphate.* The optimum concentration appeared to be nearer 0·1 than 0·2M (Fig. 3). With lower concentrations, although the reaction was accelerated in the early stages, the rate fell off rapidly because of the low pH developed as a result of reduction of tetrathionate to thiosulphate (Pollock & Knox, 1943). In the experiments given in Table 1 and Fig. 1, however, 0·2M was used rather than 0·1M phosphate in spite of the somewhat slower reaction, because the pH did not fall appreciably until near the end. (4) *Tetrathionate.* 0·02M was found in practice to be sufficient for maximal adaptation.

The part played by the phosphate, tetrathionate and mannitol separately and together was investigated. Fig. 4 illustrates a typical experiment. It consisted of two parts.

(1) A bacterial suspension prepared from a culture grown on nutrient agar was treated in five different ways, by incubation at 37° for 3 hr. in the presence of (*A*) phosphate, tetrathionate and mannitol, (*B*) phosphate and tetrathionate, (*C*) phosphate and mannitol, (*D*) phosphate and (*E*) '¼ Ringer'. Samples from the tubes containing tetrathionate (*A* and *B*) were titrated with 0·005N-I_2 (curves *A* and *B*). The contents of all five tubes were then brought to pH 7·6, kept at 4° overnight and centrifuged. (2) The resuspended deposits were then all incubated alike with

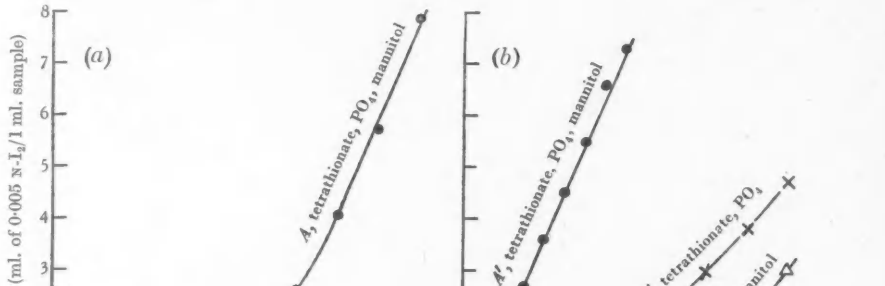

Fig. 4. Treatment of bacterial suspension in different ways. (*a*) During treatment: for explanation see text. (*b*) After treatment.

phosphate, tetrathionate and mannitol and samples titrated with 0·005 N-I_2 in the usual way (curves *A'*, *B'*, *C'*, *D'*, *E'*). Complete and rapid adaptation occurred only when both tetrathionate and mannitol were present during treatment (*A*). Incubation with '¼ Ringer' alone, followed by centrifuging, greatly delayed the adaptation process (*E'*); so did incubation with phosphate alone, though to a less degree (*D'*). Incubation with mannitol and phosphate produced a suspension (*C'*) which then adapted itself more rapidly than a suspension incubated with '¼ Ringer' or phosphate, but much more slowly than the original suspension incubated with tetrathionate and mannitol (*A*). A suspension incubated with tetrathionate and phosphate alone showed during that treatment a slow adaptation (*B*) and after treatment a further slow adaptation (*B'*) with a rate which was still not linear after 160 min., whereas with the fully adapted suspension reduction of tetrathionate was rapid and linear from the start (*A*).

DISCUSSION

The total and viable counts show that during adaptation no appreciable cell multiplication occurs. It is, of course, possible that any multiplication which did occur would not be detectable because of actual disappearance of cells by lysis, but it is unlikely that multiplication and lysis would occur at exactly the same rate. It is also possible that the power to reduce tetrathionate is restricted to a very small proportion of the cells initially present, that these cells alone are capable of multiplying in the conditions of our experiments, and that the great increase in rate of tetrathionate reduction is due entirely to multiplication of those particular cells. Neither of these possibilities could be completely eliminated by the methods we have used, nor perhaps by any technique that might be devised. It is also possible to criticize the use of bacterial counts as evidence of cell growth, since in cultures in which growth is known to be occurring it is common to find cells which have divided (often more than once) but still remain joined in one unit. These units would be counted as single cells by any method of counting. Further, during the lag phase of bacterial growth, great cell enlargement is known to occur. Neither enlargement nor incomplete cell division, if they occurred during adaptation, could be detected by counts alone. Estimations of dry weight, nephelometric comparisons and direct observations of cell size, taken together with the total and viable counts, strongly suggest, however, that adaptation is not accompanied by any significant increase in either number or size of cells, and therefore depends on factors other than a simple increase in bacterial protoplasm.

Because of the rapidity of the process, its occurrence without demonstrable cell multiplication, and the rapid loss of the new property on subculture, it is evident that natural selection is not involved and that there is true adaptation in the sense used by Yudkin. There are many ways in which adaptation might occur—by synthesis of enzyme or coenzyme, progressive removal of some inhibitor, alteration in cell membrane permeability, and other physicochemical changes on the surface or inside the cell. Without further data any attempt to explain the mechanism involved would be speculative. It would even be unwise to assume at this stage that the deviations from the 'normal' curve observed when alterations are made in the composition of the suspending fluid represent genuine effects on the adaptation process itself. The facts at present available show, however, (1) that rapid adaptation can occur without demonstrable increase in bacterial protoplasm and without any added nitrogen source and (2) that the combined presence of the specific hydrogen acceptor (tetrathionate) and a suitable hydrogen donator (mannitol) is necessary to produce complete and rapid adaptation. This supports Dubos's suggestion that for maximal adaptation the mere presence of the substrate is not sufficient; it must also be actively metabolized.

SUMMARY

1. Washed suspensions of *Bact. paratyphosum* B, prepared from cultures grown on nutrient agar, when incubated at 37° with tetrathionate, phosphate and mannitol, show at first a limited power of reducing tetrathionate to thiosulphate.

2. After an initial period of ½–1 hr. a rapid and apparently logarithmic increase in rate of reduction occurs until a linear phase is reached, when adaptation appears to be complete.

3. This increase in enzymic activity occurs in the absence of added nitrogen source, and without demonstrable increase in cell numbers or cell material.

4. Cells which have developed this property, when centrifuged and resuspended in tetrathionate, phosphate and mannitol, show rapid and linear reduction of tetrathionate from the start.

5. The new property is maintained after repeated washing of the cells and storage at 4° for several weeks.

6. Optimal development of the new property seems to require the combined presence of the specific hydrogen acceptor (tetrathionate) and a suitable hydrogen donator.

It is a pleasure to thank Dr M. Stephenson for valuable suggestions, and Dr Elkins for doing the turbidity comparisons.

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Chromatographic Investigation of the β-carotene Content of Serum of the Newborn Infant

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Several workers have shown that the carotenoid level of the blood from the umbilical cord of newborn infants is considerably lower than that of the mother (Menken, 1934; Gaehtgens, 1937; Willstaedt & With, 1939; Byrn & Eastman, 1943; Hoch, 1943*a*; Lewis, Bodansky & Shapiro, 1943). On the other hand, the last three of these investigators found that the vitamin A levels are of the same order in both. However, since the serum carotenoids are a mixture of similar substances, it seemed of interest to investigate the concentration of the biologically active β-carotene in the serum of mother and baby.

EXPERIMENTAL

Collection of blood samples. Venous blood was taken from healthy women repeatedly before and after parturition. At parturition, blood was collected from the cord and from the vagina and the samples left to clot in the dark. In a few instances venous blood was oxalated and the plasma used. Contamination of vaginal samples by other fluids was avoided as far as possible; diluted samples were, however, included in this set of analyses, since no serious error was to be expected in the relative proportions of carotenoids. This error and also that due to possible hydraemia may affect the values for the concentration of total carotenoids. In several instances the protein concentration was determined to obtain an estimate of the dilution of the blood.

Method of extraction of carotenoids from serum. 1 ml. of maternal and 1–4 ml. of foetal serum were used. 1·00 ml. of serum (or plasma) was placed in a 15 ml. narrow-necked centrifuge tube and 0·15 ml. of 29% NaOH, 0·5 ml. ethanol and a few drops of light petroleum (boiling range 40–60°) were added in succession with thorough mixing after each addition. About 3 ml. light petroleum and 0·65 ml. ethanol were then added and the mixture shaken up immediately to avoid precipitation of protein, which occurs if an equal volume of ethanol is added directly to serum. Cork stoppers wetted with distilled water were used and the mixtures were shaken by hand for 5 min. The extraction was repeated twice with about 1·5 ml. light petroleum. The pooled extracts were washed three times with distilled water, transferred to a dry centrifuge tube and, after addition of 0·3–0·4 g. anhydrous Na_2SO_4, evaporated to dryness in a stream of N_2 (bath temperature 50–55°). The extract was then dissolved in *n*-hexane and transferred quantitatively to a colorimeter tube, made up to exactly 1 ml. and centrifuged (2600 r.p.m.) until completely clear.

Determination of carotenoid content of extract. The extinction of the hexane solution was measured photoelectrically, with an Ilford Spectrum violet filter no. 601. The values of the extinction were obtained by subtracting the $\log_{10}$ (galvanometer reading for the unknown) from the $\log_{10}$ (galvanometer reading for the control (*n*-hexane)). The light intensity was adjusted so as to give a reading for the control between 95 and 105 mm. on the scale. The calibration was carried out with recrystallized chromatographically homogeneous β-carotene. Solutions of this were made up in *n*-hexane. An extinction of 0·1 corresponded to 0·62 μg. β-carotene. Correction was made for the non-linearity of the galvanometer. The deviation from proportionality of concentration and extinction was found to be less than 1% in the range normally used (extinction $< 0{\cdot}45$) and greater than 2% only with extinctions above 0·8.

A photoelectric colorimeter was built on the principle of the Evelyn instrument (Evelyn, 1936) and its essential details are given here in so far as they differ from those of the Evelyn model. The light source was a 6 V. 6 c.p. car side-light bulb fed from a car accumulator (60 amp. hr.). The light intensity was varied by means of a series stud resistance. Each step altered the galvanometer readings by about 5%. The maximum current taken was 0·57 amp. No reflector was used. The colorimeter tubes were matched ordinary centrifuge tubes $90 \times 9{\cdot}5$ mm. with an internal diameter of 8 mm. The light beam was limited by a stop to illuminate an area of 10×2 mm. on the colorimeter tube. The distance from the filament to the centre of the tube was 30 mm. A Calorex glass (infra-red filter) of 1·5 mm. thickness (manufactured by Chance Bros. Ltd.) was mounted close to the bulb and the light filters could be placed immediately in front of the photoelectric cell. The latter was an 'EEL' selenium barrier layer cell (manufactured by Evans Electroselenium Ltd.). The galvanometer used (sensitivity 840 mm./μa., 2 sec. periodic time and 175 Ω coil resistance) is a standard type S.S. 6/45 of H. Tinsley and Co.

Chromatography of serum extracts. Very uniform adsorption columns of diameters up to 13 mm. were made up as follows. A 5–10 cm. length of glass tubing was drawn to a short point so as to retain the adsorbent while allowing free passage of hexane. It was filled first with a few grains of coarse $Al(OH)_3$ and then with Al_2O_3 (standardized according to Brockmann, manufactured by Savory and Moore Ltd.). Hexane was introduced to about half the height of the tube allowing the hexane to wet the Al_2O_3. A rubber tubing was attached to the upper end of the tube and with the lower end immersed in hexane slight suction was applied and the whole of Al_2O_3 lifted 2–3 cm. When the tube was tapped the Al_2O_3 settled down and the air still contained in it collected into a small bubble which escaped through the layer of Al_2O_3 and hexane.

After estimation of the total carotenoids the serum extract was evaporated to dryness, taken up in a small volume of *n*-hexane (approx. 0·03 ml.) and transferred quantitatively with *n*-hexane to an absorption column, 16–18 mm. long and 2·2 mm. in diameter. For extracts of serum from cord blood a smaller column (1·4 × 12 mm.) was used. On elution with hexane or 0·5–1% acetone in hexane, usually five coloured bands were formed in the following order (from the bottom upwards): (1) orange-yellow, mainly β-carotene (with lipoids), identified spectroscopically and by mixed chromatogram; (2) brown-orange, usually narrow and faint (in two extracts comparatively large amounts of this pigment were present and absorption maxima in *n*-hexane were observed (visually)* at 470 and 500 mμ; this pigment was possibly identical with neolycopene A (Zechmeister, LeRosen, Schroeder, Polgár & Pauling, 1943)); (3) rose-red, mainly lycopene; (4) yellow, mainly pigments (with lipoids) with absorption maxima similar to those of α-carotene at 474 mμ and of β-carotene at 452 mμ; after saponification of these pigments both maxima appeared at positions corresponding to α-carotene. A similarly anomalous position of the 447 mμ maximum of 'leaf xanthophyll' in the presence of 0·6% butter can be seen in a graph published by Miller (1937). The explanation which is given there for the anomalous extinctions, i.e. scattering of light, is not applicable to the present case, where the solutions were optically clear; (5) yellow, mainly lutein (with lipoids). In some cases a rose-red band of the same colour as lycopene was also observed closely below the band (2). Separation of (4) and (5) into more bands was obtained by development with increasing concentrations of acetone, and, with larger quantities of extract, traces of pigments other than those mentioned were observed. Chromatography of serum carotenoids has been carried out by a number of authors (Dániel & Scheff, 1935; Süllmann, Szécsényi-Nagy & Verzár, 1936; Dániel & Béres, 1936; Willstaedt & Lindqvist, 1936; Van Veen & Lanzing,

* A small direct vision grating spectroscope, manufactured by R. and J. Beck, Ltd., London, was used for the determination of absorption maxima.

Table 1. *Changes in the extinction of carotenoids produced by saponification*

(*a*) *Saponification of the serum extracts by boiling with* 60% KOH *in methanol or with* NaOH-H_2O-*ethanol*

Method of saponification	Extinction: Before saponification	Extinction: After saponification	Duration of heating (sec.)	Extinction of β-carotene fraction of: Material not saponified	Extinction of β-carotene fraction of: Saponified material	% recoveries of total carotenoids after chromatography of: Material not saponified	% recoveries of total carotenoids after chromatography of: Saponified material
KOH-methanol	0·245	0·173	20	—	—	—	—
	0·212	0·179	30	—	—	—	—
	0·202	0·149	30	0·076	0·067	97	84
	0·167	0·160	< 30	0·061	0·053	100	93
	0·164	0·140	30	—	—	—	—
	0·172	0·148	30	0·033	0·040	83	86
	0·177	0·132	< 30	—	—	—	—
	0·200	0·158	< 30	—	—	—	—
NaOH-ethanol	0·169	0·139	180	—	—	—	—
	0·143	0·122	180	0·041	0·043	96	100
	0·218	0·148	180	0·051	0·038	95	92

(*b*) *Saponification of the fractions of serum extracts obtained by chromatography*

Fraction	Method of saponification	Extinction: Before saponification	Extinction: After saponification	Duration of heating (sec.)
1. β-Carotene	KOH-methanol	0·093	0·0915	20
		0·089	0·085	20
Remaining xanthophylls, etc.		0·165	0·116	20
		0·142	0·105	20
2. β-Carotene	NaOH-ethanol	0·171	0·164	—
		0·192	0·203	—
		0·076	0·076	30
Remaining xanthophylls, etc.		0·131	0·111	120
3. β-Carotene, pure, recrystallized	NaOH-ethanol	0·302	0·302	30

1937; Lanzing, 1938; Willstaedt & With, 1938; Thompson, 1943) and reference to unknown carotenoids (corresponding to positions between bands (1) and (4)) has been made by several of them. A pigment with absorption maxima similar to those of band (2) mentioned above has been described by Willstaedt & Lindqvist (1936).

In this investigation no attempt has been made to separate the small amounts of carotenoids other than β-carotene which accompany the lipoids of the first fraction. The first coloured band was eluted with *n*-hexane and it is designated here as β-carotene. The remainder of the pigments was eluted by adding a small amount of absolute ethanol to the hexane still left on top of the column. Low recoveries may be due to incomplete elution of pigments other than β-carotene and to bleaching of all fractions, including β-carotene. I have, therefore, calculated the extinction which is contributed by fraction (1) as a percentage of the total extinction found both before and after chromatography.

Saponification of extracts. Chromatography of extracts from serum prepared without saponification may be complicated by the presence of esters of xanthophylls and by lipoids which act as eluting agents. The lipoids may also affect the extinction measurements. Chromatography is therefore usually carried out on saponified material (Zechmeister & Cholnoky, 1943). The two main reasons for the choice in this investigation of a technique employing extracts which have not been saponified are (*a*) the losses in total carotenoids observed under conditions of hot saponification (Table 1) and (*b*) the possibility of the production of artefacts.

The loss of carotenoids is mainly due to losses of lutein and carotenoids other than β-carotene. Losses of carotenoids on saponification have been previously observed by Clausen & McCoord (1936).

Recrystallized chromatographically homogeneous β-carotene showed no losses in extinction after heat treatment with alkali, but it then contained small amounts of pigment which form a yellow band below the β-carotene (probably this is neo-β-carotene B (Zechmeister & Polgár, 1942)) and some yellow pigment adsorbed above β-carotene. This pigment had a general absorption in the blue and violet from about 500 mμ. No pronounced maxima could be observed visually. A pigment similar in appearance and spectrum was also found in very small amounts in the unsaponified extract of one serum.

The saponification of the β-carotene (plus lipoid) fraction of serum extracts led to the formation of a sharp red-orange band above the β-carotene. This effect was also obtained with nearly lipoid-free, chromatographically homogeneous β-carotene isolated from serum.

The known xanthophyll esters complicate the chromatogram on the Al_2O_3 column only at positions above the lycopene (Zechmeister & Cholnoky, 1943) and therefore would not be expected to interfere with the separation of β-carotene.

In order to estimate the influence of the lipoids on the extinction measurements, the β-carotene fraction was again chromatographed on an activated Al_2O_3 type H 100/200 (manufactured by Peter Spence and Sons, Ltd.), which is a very strong adsorbent, and the bulk of the lipoids were separated. The β-carotene which appeared orange-yellow when first adsorbed, later changed to pink and after elution with acetone-hexane had the same absorption maxima as crystallized β-carotene. When the lipoid fraction was again united with this material the 478 mμ maximum was shifted towards the longer wave-lengths and the solution appeared slightly more orange than pure β-carotene. The effect of lipoids on the extinction in a sample containing 1·2 μg. β-carotene in 1 ml. *n*-hexane as measured with the Spectrum violet filter was, however, small and well within the experimental error (+0·5%). It seems fair to assume that the error in the estimation of β-carotene caused by the presence

Table 2. *Comparison of the photoelectric and visual (micro-) estimation of carotenoids*

(μg. carotenoid, as β-carotene.)

	Photo-electric	Visual	Ratio: visual / photoelectric
Total carotenoids	0·162	0·162	1·00
	0·493	0·490	0·99
	0·083	0·097	1·17
	0·205	0·222	1·08
	0·188	0·198	1·05
	0·244	0·226	0·93
	0·324	0·331	1·02
	0·470	0·518	1·10
	0·580	0·566	0·98
Fraction (1), β-carotene	0·050	0·052	1·04
Fractions (2)–(5)	0·262	0·256	0·98
Fraction (1), β-carotene	0·127	0·12	0·95
Fractions (2)–(5)	0·787	0·76	0·96
		Average	1·02

Table 3. *Carotenoids, β-carotene and total proteins in venous serum at different times before and after parturition, and in serum of blood from the vagina at parturition*

Subject	Date	Total protein (g./100 ml.)	Total carotenoids (μg./100 ml.)	% β-carotene of total: After chromatography	% β-carotene of total: Before chromatography
A.W.	4. ii. 44	6·71	142	33·5	31·0
	13. iii. 44	6·66	186	40·5	37·5
	27. iv. 44	6·56	196	37·0	36·5
	7. v. 44 (parturition)	6·06	163	39·5	38·5
	9. v. 44	6·01	154	38·5	35·5
	26. v. 44	6·83	138	39·5	37·0
H.S.*	10. xi. 43	—	123	30·0	34·0
	1. xii. 43	—	100	31·0	27·5
	15. xii. 43	—	121	24·5	22·0
	29. xii. 43	—	144	35·5	35·5
	18. ii. 44	—	175	33·5	31·5
	23. iii. 44	—	199	25·5	25·5
	18. iv. 44	6·48	278	28·5	27·5
	21. iv. 44 (parturition)	6·43	249	29·5	28·5
	27. iv. 44	—	163	34·0	32·0
	4. v. 44	7·44	184	32·5	31·5
D.S.	30. xii. 43	6·77	91	40·0	35·5
	9. xii. 43	6·75	134	42·0	39·5
	23. iii. 44	6·23	131	34·0	31·0
	3. v. 44 (parturition)	—	138	37·5	36·0
	5. v. 44	6·15	128	38·5	37·5

* First six samples taken by finger prick and analyzed by the visual method using 0·157 ml. serum.

of serum lipoids will be negligible provided the wave band used is not too narrow and measurements are made at a sufficient dilution.

Microtechnique for determination of serum carotenoids. Total carotenoids from cord blood serum were determined both photoelectrically and visually (Hoch, 1943*b*), while the chromatographic fractions were measured visually since the concentrations were below the limit of the photoelectric method. In two cases it was possible to measure these fractions also photoelectrically (see Table 2) and the proportions of β-carotene found by the two methods were 16 and 17% in one, and 14 and 13·5% in the other case. A serious drawback of the visual micro-estimation of serum carotenoids in extracts prepared without saponification—besides the low accuracy inherent in a visual method which employs artificial standards—is the difference in colour of the β-carotene fraction due to the lipoids present (see above), which is pronounced if the lipoids form an appreciable portion of the total volume (< 0·04 ml.) used. Table 2 shows the results on samples estimated by both photoelectric and visual method.

RESULTS

Table 3 shows the levels of carotenoids, β-carotene and proteins in maternal serum at different times before, at and after parturition.

The variation in the proportion of β-carotene near term was small and did not exceed 20% (see also Table 5).

Tables 4–6 show the relation of the total carotenoids, β-carotene, lycopene and pigment (2) in serum of mother and infant at parturition.

The concentrations of total carotenoids found in cord serum are in agreement with earlier work (Menken, 1934; Gaehtgens, 1937; Willstaedt & With, 1939; Byrn & Eastman, 1943; Lewis, Bodansky & Shapiro, 1943; Hoch, 1943*a*).

The proportion of β-carotene in total carotenoids was markedly lower in the foetal than in maternal

Table 4. *Total carotenoid content of maternal and foetal serum*

(The figures in brackets refer to venous blood 2–4 days after parturition.)
Carotenoid content expressed as β-carotene (μg./100 ml. serum).

	M.H.	R.	J.F.	E.S.*	F.L.	K.J.	H.S.	D.S.	A.W.
Mother	62 (92)	80 (92)	53 (158)	21 (116)	107 (89)	230 (183)	249 (163)	138 (128)	160 (154)
Infant	13	18	18	8	9	24	29	26	17
Ratio $\frac{\text{infant}}{\text{mother}}$	0·21	0·23	0·34	0·38	0·08	0·10	0·12	0·19	0·11

* Blood sample much diluted.

Table 5. *Proportions of β-carotene in the total carotenoids of maternal and foetal serum*

(*a*) % β-carotene, of total carotenoids recovered after chromatography. (*b*) % β-carotene, of total carotenoids measured before chromatography.

	M.H.		R.		J.F.		E.S.		F.L.	
Mother:	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
At parturition	35	32 (91)	20·5	15 (72)?	39·5	37 (93·5)	42·5	44·5 (104)	45	43 (95)
2–4 days after parturition	40·5	39·5 (97)	21	20 (95)	38	35·5 (93·5)	52	51·5 (99)	47	44·5 (95)
Infant at parturition	20	19·5 (98)	13·5	12·5 (93)	22	17·5 (80)	30·5	28·5 (93)	24	23·5 (98)
Ratio $\frac{\text{infant}}{\text{mother}}$	0·57		0·66		0·56		0·72		0·53	

	K.J.		H.S.		D.S.		A.W.	
Mother:	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
At parturition	34·5	33 (95)	29·5	28·5 (97)	38	36·5 (96)	39·5	38·5 (98)
2–4 days after parturition	36	34·5 (96)	34	32 (94)	39	38 (97)	38·5	35·5 (92)
			6 days after parturition					
Infant at parturition	20	16·5 (84)	16	14·5 (91)	14	12·5 (89)	16	15 (94)
Ratio $\frac{\text{infant}}{\text{mother}}$	0·58		0·54		0·37		0·40	

(The figures in brackets give the recoveries of total carotenoids after chromatography.)

Table 6. *Amounts of brown-orange pigment* (2) *and lycopene* (3) *in maternal and foetal serum*

	M.H.		R.		J.F.		E.S.		F.L.		K.J.		D.S.		A.W.	
	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)
Mother	+	Trace	+	+ + +	0	+ +	+	0	0	+	+ + +	+ +	+ +	+ +	+	+
Infant	+	0	0	+ +	0	+ +	?	0	0	0	+ +	+	0	Trace	0	0

serum in all cases investigated. The ranges were 13·5–30·5 % for foetal and 20·5–42·5 % for maternal serum at parturition. The ratio

$$\frac{\%\ \beta\text{-carotene in total carotenoids (infant)}}{\%\ \beta\text{-carotene in total carotenoids (mother)}}$$

was between 0·53 and 0·72 in seven, and 0·40 and 0·37 in two cases. Pigment (2) and lycopene, when present in maternal serum, appeared also in foetal serum in a number of instances. In four instances pigment (2), and in three instances lycopene, was not found in foetal serum, although contained in maternal serum.

DISCUSSION

The difference between the concentrations of total carotenoids of maternal and foetal serum may be explained by assuming a barrier in the placenta, since it is improbable that there is a mechanism in the foetus sufficiently effective to remove the carotenoids from the circulation at the rate at which they would enter it if there were no such barrier.

The difference in the proportions of β-carotene in the carotenoids of mother and infant can be interpreted as the resultant of two functions: (*a*) the permeability of the placenta and (*b*) the rate of elimination from the foetal circulation. These are probably different for each individual carotenoid. It is remarkable that the ratio

$$\frac{\%\ \beta\text{-carotene in total carotenoids (infant)}}{\%\ \beta\text{-carotene in total carotenoids (mother)}}$$

varies only within fairly narrow limits. One may suppose that a constant ratio would not be brought about by placentae of different permeabilities and that more probably the foetal organism would be mainly responsible for the smaller proportion of β-carotene in total carotenoids of cord blood serum, e.g. by converting it into vitamin A, or storing it, more quickly than the xanthophylls are being disposed of.

SUMMARY

1. The total carotenoids and the proportion of β-carotene in the total carotenoids were determined in maternal and foetal serum at parturition. The total carotenoids in foetal serum ranged from 8 to 29 μg./100 ml. The proportion of β-carotene in the total carotenoids was markedly lower in the infant's serum in all cases. The ratio

$$\frac{\%\ \beta\text{-carotene in total carotenoids (infant)}}{\%\ \beta\text{-carotene in total carotenoids (mother)}}$$

was 0·53–0·72 in seven, and 0·40 and 0·37 in two cases.

2. There was no significant variation of the percentage β-carotene of total carotenoids in the maternal serum during the time near term.

3. A micro chromatographic technique for the separation of the carotenoids of serum is described.

4. The use of extracts without saponification for the determination of serum carotenoids is advocated.

5. Details of construction of an instrument similar to the Evelyn photoelectric colorimeter are given.

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The Reaction between Histidine and Formaldehyde

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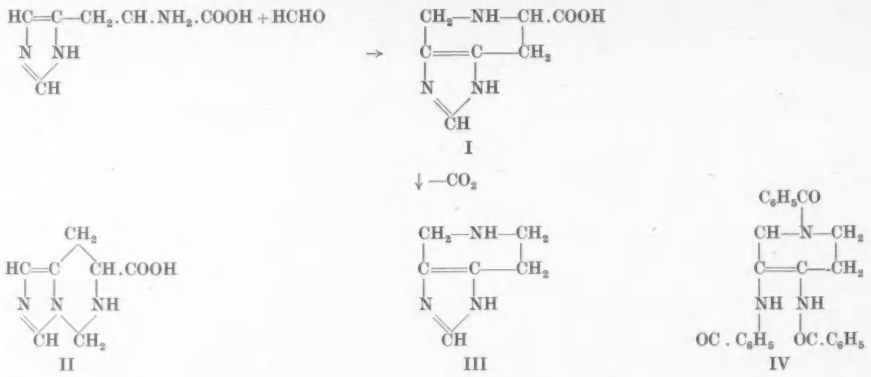
The reaction between formaldehyde and amino-acids occurs in two distinct stages (Wadsworth & Pangborn, 1936). The first stage is a reversible reaction between the carbonyl and amino groups leading to compounds of an aldimine or *N*-methylol structure which dissociate readily into the original components. This reaction has been extensively studied by potentiometric and polarimetric methods. The second phase consists of reactions which are irreversible in most cases and the nature of which varies for different amino-acids. This second reaction is very slow in some cases, e.g. with glutamic acid where no reaction products have so far been isolated; in others it is, however, quite fast, as with asparagine which leads to a hexahydropyrimidine derivative (Schiff, 1900; Levy & Silbermann, 1937).

The reaction between histidine and formaldehyde has been investigated by several workers. Birch & Harris (1930) and Levy (1935*a*) have studied the reaction potentiometrically and interpreted their results in terms of a reversible association of formaldehyde and the amino-acid; the latter author assumed that only the α-amino group was involved. Frieden, Dunn & Coryell (1943), who used a polarimetric method, also considered the reaction to be reversible. That such an assumption, however, is incorrect had already been shown by Holden & Freeman (1931), who demonstrated that, if histidine is incubated with about 2·5 mol. of formaldehyde at 37°, most of the α-amino-N, as measured by the Van Slyke method, is lost in the first few minutes. These authors also noticed that an insoluble product crystallized from the reaction mixture; this compound was not further investigated. The very fast, irreversible reaction of histidine was later confirmed by Wadsworth & Pangborn (1936). The present paper deals with the structure of the compounds formed, their dissociation constants and the mechanism of the reaction.

If histidine is incubated at 37° with two or more mol. of formaldehyde, a very insoluble compound (*A*) is formed which contains two more carbon atoms than histidine. This compound dissociates on heating or on acidification, and splits off one mol. of formaldehyde. A new stable compound (*B*) is formed which contains only one carbon atom more than histidine. Compound *B* can also be obtained by incubating histidine with exactly one mol. formaldehyde, or by treatment of histidine with methylal and conc. HCl at 100° (Wellisch, 1913). On addition of one mol. of formaldehyde, compound *B* is converted almost instantaneously into compound *A*. These reactions and analysis show that compounds *A* and *B* have the same fundamental structure and compound *A* is only an *N*-methylol derivative of *B*.

Compound *B* gives a negative ninhydrin reaction and does not react with nitrous acid with evolution of gas. Analysis indicates that its formula is $C_7H_9O_2N_3$. It appears, therefore, that a new ring has been formed involving both the α-amino group and the iminazole nucleus. The new ring could have either a tetrahydropyridine structure (I), formed by interaction with C_4 of the iminazole nucleus or a hexahydropyrimidine structure (II) involving the imino-group (N_1). Formation of a seven-membered ring by combination with the C_2 seems unlikely. The stability of *B* to acid and alkali appears to exclude a hexhydropyrimidine structure, since fully reduced pyrimidines are very easily hydrolyzed by acid (Titherley & Branch, 1913). Both *A* and *B* give positive Pauly reactions; this reaction is not given by N-substituted iminazoles (Burian, 1904), whilst 4:5-disubstituted derivatives give a positive reaction (Fargher & Pyman, 1919). The positive Pauly reaction is, therefore, in accordance with structure (I).

More direct evidence was, however, obtained in the following way: *B* was decarboxylated, though in bad yield, at 265° in fluorene (Waser, 1925) to a base (III) which was found to be identical with a compound obtained by condensation of histamine with methylal (Fraenkel & Zeimer, 1920; Dale & Dudley, 1921). This base, on benzoylation, gave formic acid and a crystalline compound which analyzed correctly for a tribenzoyl derivative of diamino-tetrahydropyridine (IV). The formation of a tribenzoyl compound indicates that a Bamberger-Berlé fission has occurred during benzoylation. Since N-substituted iminazoles do not react with benzoyl chloride and alkali (Pinner & Schwarz, 1902), *B* cannot have structure (II); likewise the formation of formic acid and the isolation of (IV) excludes a seven-membered ring structure.



The compounds obtained from histidine by condensation with formaldehyde are optically active; the decrease in laevo-rotation on addition of acid indicates, according to the criteria established by Lutz & Jirgensons (1930), that the asymmetric centre in (I) has the *l*-configuration. It may, therefore, be designated *l*(−)1:2:5:6-*tetrahydropyrido*-3:4-*iminazole*-6-*carboxylic acid*. Compound *A* also has the tetrahydropyrido-iminazole structure, but the position of the labile methylol group is, however, uncertain; it may be situated at either of the two imino-nitrogens in (I), as shown in formulae (V) and (VI) respectively.

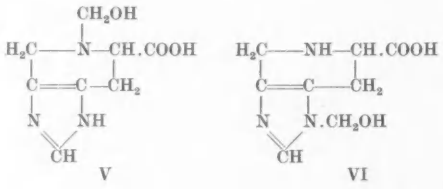


Table 1. *Apparent dissociation constants of histidine and of* 1:2:5:6-*tetrahydropyrido*-3:4-*iminazole*-6-*carboxylic acid in the absence and presence of formaldehyde at* 25°

The values for histidine are those of Levy (1935*b*), recalculated for 25°. The other values were obtained with solutions of an ionic strength of approximately 0·08. No extrapolation to zero ionic strength was made.

	Substance	pK_2	pK_3
1	Histidine	6·12	9·27
2	1:2:5:6-Tetrahydropyrido-3:4-iminazole-6-carboxylic acid	4·73	8·58
3	Do. + 1·05 mol. of formaldehyde	4·56	8·35
4	Do. + 2·5 mol. of formaldehyde	4·43	8·21
5	Do. + 20 mol. of formaldehyde	4·15	7·51

In view of the possibility that compounds *A* and *B* may be largely responsible for the behaviour of histidine under the conditions used in the Sorensen formaldehyde titration of amino-acids, the apparent dissociation constants of *B* and the changes of these dissociation constants on addition of formaldehyde were determined. Table 1 gives the pK values of the basic groups of histidine and of *B*. pK_2 corresponds to the ionization of the iminazole nucleus, whilst pK_3 is due to the ionization of the α-amino group of histidine or of the tetrahydropyridine nitrogen of *B*. It can be seen that the pK of the iminazole group has decreased in *B* as compared with that of histidine by about 1·4 units; this is mainly due to the close proximity of the positively charged second imino group which is now separated from the iminazole nucleus by only one methylene group. The change in pK_3 is not quite so marked, being only about 0·7 unit; the decrease of this pK may be due to the electronegative character of the double bond between C_4 and C_5 of the iminazole group having a greater effect in the tetrahydropyridine ring than in the open chain system of histidine.

On addition of increasing amounts of formaldehyde, both pK values decrease progressively, but the shift is greater in pK_3. These changes can be interpreted as indicating a reaction of both imino groups with the aldehyde; but it is also possible that formaldehyde combines only with one imino group and the change in the other pK is due to the effect of the substitution of hydrogen by the electronegative methylol group on the other imino group. Potentiometric data do not enable us to decide between these two alternatives, but the fact that *A* contains only one methylol group favours the second interpretation.

The optical rotations of *B*, its salts and that of the sodium salt of *A* are very high compared with those of histidine and its salts (*A* itself is too insoluble to allow its rotation to be measured). Observation of changes of rotation with time appeared, therefore, to afford a convenient method of following the kinetics of the condensation. Fig. 1 shows the

rotations observed over the first 48 hr. at different pH's. The values are not strictly comparable, since the rotations of both histidine and the condensation products vary with pH. It is clear, however, that the changes in rotation are very fast at pH values above 5·0. The speed does not increase appreciably on raising the pH from 7·4 to 11·2. Below pH 4·0 the reaction is very slow. The final values which are obtained after 36–96 hr. agree within 5% with slower increase which is not quite finished after 48 hr. This apparent slowing down of the reaction cannot be caused by a shift of pH occurring during the condensation, since it is also found in a buffered solution. Nor can it be due to slow depolymerization of formaldehyde or a fixation of formaldehyde by the reaction products, since the shape of the curve is not greatly affected by an increase in the proportion of the aldehyde. It is probable there are

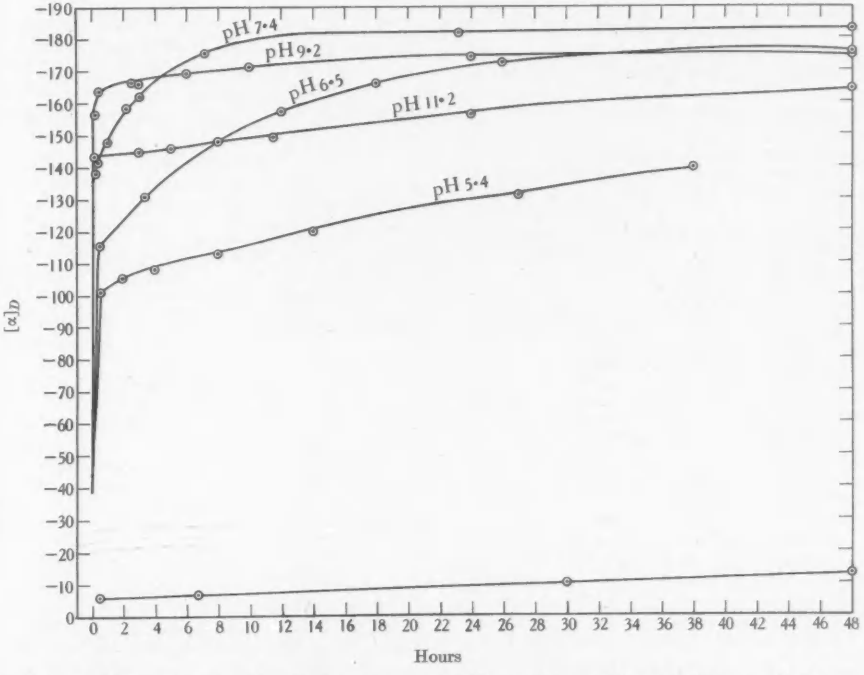


Fig. 1. Changes of optical rotation of equimolar mixtures of histidine and formaldehyde at different pH's. The rotations are expressed in terms of $[\alpha]_D$ of histidine. The pH stated refers to the initial pH of the histidine-buffer mixture before formaldehyde was added. The required pH was obtained by adding the calculated amount of acid or alkali to the histidine solution, which was then mixed with the appropriate buffer; 0·2M acetate, phosphate and borate respectively were used. The molar concentration of the buffer greatly exceeded that of the histidine.

those calculated for the respective condensation products at the particular pH used. It might appear from this, and from the fact that *A* and *B* respectively can be isolated in crystalline form from these mixtures after adjustment of pH to about 6·6, that the changes in rotation are simply a measure of a direct conversion of histidine to these two products, but closer inspection of the curves (Fig. 2) does not bear out this interpretation. The very rapid change which occurs in the first 3 min. and which is equivalent at pH 7·4 to about half the total change in rotation observed, is followed by a very much two reactions involved, a first fast reaction being followed by a much slower one, the latter leading to compounds *A* and *B*. The failure to isolate *A* and *B* during the first few hours of the reaction, even after inoculation, supports this explanation. Wadsworth & Pangborn (1936) drew the same conclusion that this reaction is a two-stage process from a comparison of the disappearance of amino-N and of free formaldehyde. They observed that, whilst loss of amino-N as measured by the nitrous acid method was very fast, as already found by Holden & Freeman (1931), the disappearance of formaldehyde,

as estimated by the dimedon method, was very much slower. They concluded that the initial product is more stable towards nitrous acid than towards dimedon. On the nature of the first reaction one can only speculate. It might be a Schiff's base or mono- or dimethylol compound formed by reaction with the α-amino group. It would have to be assumed that the hypothetical intermediate, although unstable towards dimedon, is not split nitrous acid and hydrolyzed in the presence of dimedon, due to the reversible nature of this condensation (Titherley & Branch, 1913). Such a rearrangement involving a migration from nitrogen to carbon would be similar to that postulated by Pauly (1915) in the coupling of iminazoles with diazonium salts.

The question naturally arises as to the relation of these results to those of the titration of histidine

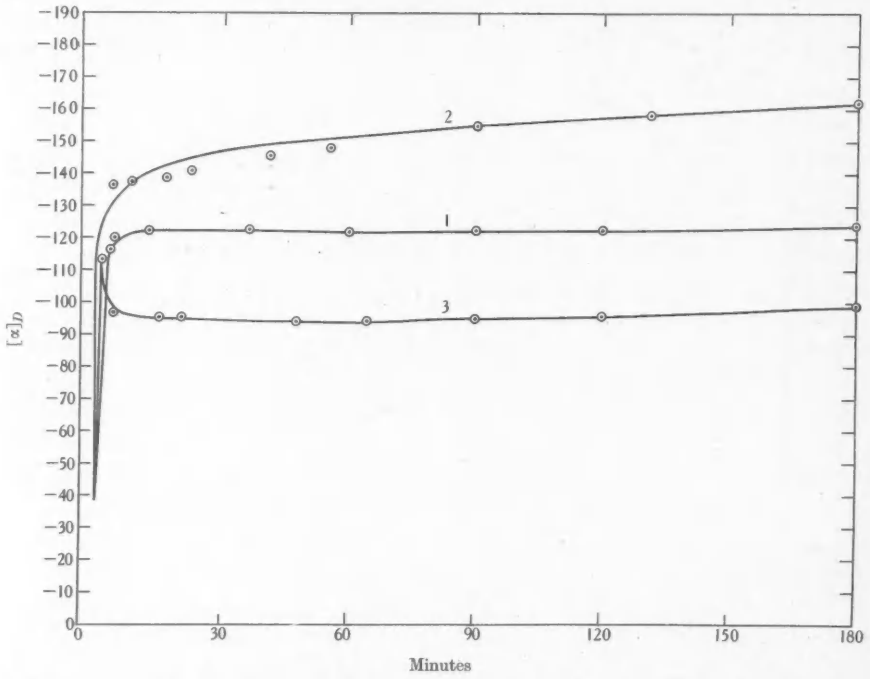


Fig. 2. Changes of rotation of mixtures of isoelectric histidine with 1 or 2 mol. of formaldehyde. Experimental details as in Fig. 1. Curve 1: 1 mol. of formaldehyde, unbuffered. Curve 2: 1 mol. of formaldehyde, phosphate buffer of pH 7·4. Curve 3: 2 mol. of formaldehyde, unbuffered.

under the acid conditions used in the nitrous acid method. Such an assumption implies that the first reaction product differs in its stability from analogous formaldehyde derivatives of many other amino-acids which are easily split and give full amino-N values. It is possible that the positively charged iminazole group might stabilize somewhat an N-methylol linkage, without affecting its reactivity with dimedon, although evidence on this point is lacking. Another possibility is that the initial product has the hexahydropyrimidine structure (II), which then rearranges itself to a tetrahydropyridine (I). It might be expected to be stable to by alkali in the presence of formaldehyde by the Sorensen method. A solution of histidine and formaldehyde shortly after mixing will contain, besides compound *A* or *B*, a considerable proportion of the initial product whose existence, as pointed out above, is only inferred and whose nature is the subject of discussion. The titration data of Levy (1935*a*) suggest an initial cyclization, involving iminazole groups. As Levy (1935*a*) found, the *pK* of iminazoles carrying no aliphatic amino group is not shifted by addition of formaldehyde, whilst in histidine the *pK* values of both the amino and the iminazole groups are displaced by formaldehyde.

Such displacement is most easily explained by assuming that the iminazole group is involved in the initial reaction.

The wide pH range, over which the reaction takes place, suggests that condensation does not depend on the amino group and probably not even on the iminazole group being uncharged. Acylation of the amino group, however, prevents condensation, since both α-N-benzoyl and α-N-acetyl-histidine were recovered unchanged on prolonged incubation with excess of formaldehyde at 37°. The reaction investigated here can, therefore, have no direct application to the interaction of formaldehyde with proteins, unless we assume that some of the α-amino groups present in proteins are those of histidine, but it is possible that methylene bridges may be formed by formaldehyde between iminazole groups in proteins and neighbouring amino groups.

Another point must be mentioned. The rapid reaction of histidine with formaldehyde and possibly with other aldehydes may lead to errors in the estimation of β-hydroxy-α-amino-acids by the periodate method, especially in the estimation of serine, which is based on the measurement of the formaldehyde evolved (Nicolet & Shinn, 1939; Boyd & Logan, 1942).

EXPERIMENTAL

All m.p.'s are uncorrected. Elementary micro-analyses were kindly carried out by Mr Parker.

All pH measurements were made at 25° ± 0·1 with a glass-electrode which had been standardized against phthalate and phosphate buffers.

Polarimetric measurements were carried out at concentrations of histidine varying between 1·9 and 2·1%. The temperature varied between 18 and 21°.

Preparation of compound A *from histidine.* A mixture of *l*(−)-histidine (3·1 g.) dissolved in water (80 ml.) and 6M-formaldehyde solution (8·5 ml.) was left at 37° for 40 hr. The crystalline precipitate was filtered off and washed successively with water, ethanol and ether (yield, 95%); it had m.p. 210–215° (decomp.). The substance contains water of crystallization which is lost on drying at 100°/0·1 mm. (Found: C, 44·4; H, 6·2; N, 19·6; loss on drying, 8·3. $C_8H_{11}O_3N_3.H_2O$ requires C, 44·6; H, 6·1; N, 19·5; H_2O, 8·4%.) The dried material is very hygroscopic.

The substance is insoluble in cold water and in all the usual organic solvents. On heating in water it went into solution slowly, and a distinct smell of formaldehyde became noticeable; on cooling and standing, about 80% of the original material of m.p. 210–215° crystallized out again. The substance dissolved on addition of one equiv. of HCl, with liberation of formaldehyde which could be precipitated as its dimedon derivative. On neutralizing of the acidified solution, a precipitate again appeared which was shown to be identical with the original material by crystalline form, m.p., N- and water-content. The substance also dissolved in alkali; a solution in 1·1 equiv. of NaOH had $[\alpha]_D = -84·6°$ ($c = 1·6$ in terms of free base).

Pauly's reaction was positive, even when the alkaline solution of compound *A* was added to the alkaline, diazotized solution of sulphanilic acid. The intensity of the colour was about equal to that given by an equimolar solution of histidine. The ninhydrin reaction was negative.

Preparation of compound B. (1) *By hydrolysis of compound* A. To a solution of compound *A* (1·2 g.) in water (50 ml.) containing 2N-HCl (3 ml.), dimedon (2·2 g.) dissolved in warm water (200 ml.) was added. The mixture was left for 48 hr. at room temperature. The precipitate of formaldehyde-dimedon compound which had formed was collected and weighed. Recrystallized from ethanol it had m.p. 188–189° undepressed by an authentic specimen. (Yield, 98% of theoretical for loss of 1 mol. formaldehyde.) The mother liquor and washings were combined, neutralized exactly with LiOH and concentrated *in vacuo*. On addition of ethanol, large needles separated. (Yield, 60%.) On recrystallization from a little water, m.p. was 277°. (Found: C, 41·6; H, 6·3; N, 20·6; loss on drying, 17·2. $C_7H_9O_2N_3.2H_2O$ requires C, 41·4; H, 6·4; N, 20·7; H_2O, 17·8%.) The substance contained water of crystallization which could be removed by drying at 100°/0·1 mm. The Pauly reaction was positive. The ninhydrin reaction and Knoop's bromine reaction were negative. The $[\alpha]_D$ values at different pH's were as follows:

Addition	$[\alpha]_D$	Concentration (%)
—	−144·5°	2·2
1 equiv. NaOH	−122·4°	2·1
1 equiv. HCl	−139·7°	1·85
2 equiv. HCl	−121·2°	1·6

(2) *From histidine of* pH 7·45. *l*(−)-Histidine (0·465 g.) dissolved in water (10 ml.) was incubated with exactly 1 mol. of formaldehyde (3 ml. of 1N-solution) at 37° for 2 days. The solution was then chilled to 0° and the crystalline precipitate filtered off. It was recrystallized from aqueous ethanol and shown to be identical with compound *B* by analysis and optical rotation.

(3) *From histidine by condensation with methylal and conc.* HCl. *l*(−)-Histidine monohydrochloride hydrate (5·6 g.) was dissolved in conc. HCl (24 ml.) and the solution kept at 100° for 3·5 hr., whilst methylal (5 ml.) was slowly added through a dropping funnel. After having been kept at that temperature for a further 4 hr., the solution was evaporated *in vacuo*. The residue was taken up in water, the pH adjusted to 3·5 by addition of pyridine and ethanol was added. The precipitate was collected and recrystallized from aqueous ethanol; it had m.p. 279–280°. (Yield, 75%.) (Found: C, 41·0; H, 5·1; N, 20·7; Cl, 17·3. $C_7H_{10}O_2N_3Cl$ requires C, 41·3; H, 4·9; N, 20·6; Cl, 17·4%.) The hydrochloride was converted into compound *B* itself by exact neutralization of the concentrated aqueous solution with LiOH. The free base was identified by analysis, water content and optical rotation.

Conversion of compound B *into* A. Compound *B* (0·215 g.) was dissolved in water (5 ml.) and 6M-formaldehyde solution (0·25) was added. Large rod-like crystals appeared almost at once. The material had m.p. 210–215° and $[\alpha]_D = -85°$ ($c = 2·0$ in 1·1 equiv. of NaOH). (Found: N, 19·3; calc. for $C_8H_{13}O_4N_3$: N, 19·5%.) (Yield, 90%.)

Attempted condensation of α-*N-benzoyl-histidine with formaldehyde.* α-*N*-Benzoyl-*l*(−)-histidine (8·13 g.) was dissolved in water (100 ml.) containing 1·08N-LiOH (27·2 ml.) and formaldehyde (10 equiv.). The mixture was left at 37° for 10 days. N-HCl (29 ml.) was then added. The solution was then concentrated *in vacuo* to dryness, the residue was dissolved in water and again concentrated. On addition

6·5 g. of crystalline material were obtained which was found to be unchanged hydrate of benzoyl-histidine. It had m.p. 245° (decomp.) and $[\alpha]_D = -47·1°$ ($c = 2·1$). The authentic specimen had m.p. 247° (decomp.) and $[\alpha]_D = -47·3°$ ($c = 2·2$).

Decarboxylation of compound B *to* 1:2:5:6-*tetrahydropyrido*-3:4-*iminazole*. Compound *B* (1·5 g.) was heated to 130° until all the water of crystallization had been removed and was mixed with 4·5 g. of melted fluorene. The mixture was then slowly heated to 265° and kept between 265° and 270° for 30 min. The mixture was cooled, extracted twice with 15 ml. 2N-HCl, the acid solution extracted with ether and then made strongly alkaline by addition of 10N-NaOH. The alkaline solution was extracted with amyl alcohol (150 ml.) in three portions, the combined alcoholic extracts dried over Na_2SO_4, filtered and extracted five times with N-HCl. The combined acid extracts were evaporated *in vacuo* and the residue recrystallized twice from 95% aqueous ethanol. It amounted to 35 mg. and had m.p. 276–277°. A specimen mixed with material prepared from histamine (Dale & Dudley, 1921) showed no depression. (Found: N, 21·2. Calc. for $C_6H_{11}N_3Cl_2$: N, 21·47%.) 25 mg. were converted into the picrate, which gave m.p. 212–214° alone or mixed with the dipicrate of the product from histamine.

Preparation of 3:4-*dibenzamido*-N-*benzoyl*-1:2:5:6-*tetrahydropyridine*. 1:2:5:6-Tetrahydropyrido-3:4-iminazole (0·9 g.) prepared from histamine (Dale & Dudley, 1921) was dissolved in N-NaOH (10 ml.) and benzoylated with benzoylchloride (4 g.) and 2N-NaOH (16 ml.) in the usual way. The oil which formed during the reaction was extracted with chloroform, the extract washed successively with N-NaOH, water and 2N-HCl, dried and evaporated *in vacuo*. Twice crystallized from 60% aqueous ethanol, the product had m.p. 215°. (Yield, 25%.) (Found: C, 73·2; H, 5·2; N, 10·1. Calc. for $C_{26}H_{23}O_3N_3$: C, 73·4; H, 5·4; N, 9·9%.)

The mother liquor from the benzoylation was acidified and most of the benzoic acid removed by filtration. The solution was then steam-distilled. The distillate was made just alkaline with NaOH and concentrated to small volume. The residue was then acidified with H_2SO_4 and again distilled. The distillate was strongly acid, although apparently free from H_2SO_4, and reduced $AgNO_3$ under acid conditions.

SUMMARY

1. It is shown that histidine reacts with one mol. of formaldehyde to give a tetrahydropyrido-iminazole carboxylic acid. The structure of this acid has been demonstrated by decarboxylation followed by a Bamberger-Berlé fission. In presence of excess formaldehyde a very insoluble methylol derivative of this product is obtained.

2. The dissociation constants of the two compounds have been measured and compared with those of histidine.

3. The kinetics of the reaction have been examined and the bearing of the results on the formaldehyde titration of histidine been discussed.

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The Microbiological Assay of Nicotinic Acid in Cereals and Other Products

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We have had difficulty in the assay of nicotinic acid in cereals and cereal products by the chemical method described by Kodicek (1940), owing to the presence of interfering substances. On the other hand, the microbiological method originally introduced by Snell & Wright (1941), later modified by Krehl, Strong & Elvehjem (1943), and now further modified, has been found to be expeditious and accurate.

The chief drawback to the Snell & Wright method as originally published was the non-linearity of the standard curve with concentrations of nicotinic acid

above 0·15–0·2 μg./10 ml. This difficulty was overcome by Krehl *et al.* who raised the concentration of glucose and buffer (sodium acetate) to 2%. This increase in concentration of glucose and buffer led to an increase in acid production and enhanced the linearity of the standard curve. Further modifications introduced by Krehl *et al.* were to double the concentration of cystine and halve the concentration of biotin (i.e. 0·2 μg. instead of 0·4 μg. biotin/1000 ml. of medium). In the present investigation the modifications that have been found to give the best results in addition to those described by Krehl *et al.* are: (1) to double the concentration of casein hydrolysate (1% instead of 0·5%), (2) to maintain the concentration of biotin at 0·4 μg./1000 ml. medium, (3) to add xanthine and (4) to add 0·1% xylose.

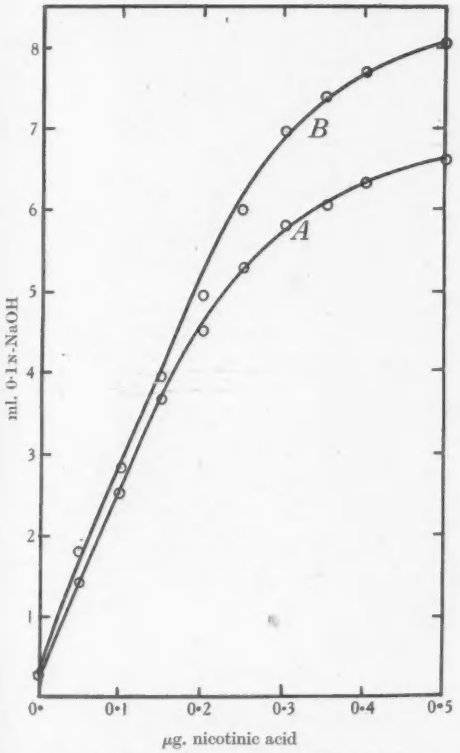

Fig. 1. *A*. Standard curve obtained on original Snell and Wright medium. *B*. Standard curve obtained on modified medium with 2% glucose and 2% sodium acetate.

Fig. 1 shows a standard curve (*A*) obtained in the original Snell & Wright medium and a standard curve (*B*) obtained on the modified medium described here.

A difficulty encountered with the method in the past has been the incidence of high blanks, due to three main causes: (i) it was difficult to rid the casein hydrolysate of the last traces of nicotinic acid; (ii) natural *l*-tryptophan, unless carefully purified, is liable to be heavily contaminated with nicotinic acid. One sample of natural *l*-tryptophan was found to contain as high a concentration as 20 μg./g. of nicotinic acid, which will vitiate any microbiological assay; (iii) the crude concentrates of biotin which were at one time the only available sources of this vitamin were frequently contaminated with nicotinic acid. This last difficulty no longer exists, as pure crystalline biotin is now available.

EXPERIMENTAL

Organism. The organism employed was *Lactobacillus arabinosus* 17/5 from Snell & Wright's original strain obtained from the American Type Culture Collection, Georgetown University, Washington, D.C. Stock cultures of the organism were carried on a yeast-water-glucose agar to which had been added 0·6% of sodium acetate. The cultures were preserved in a refrigerator at a temperature of 4° approx. and renewed at fortnightly intervals.

Composition of basal medium. This is given in Table 1.

Table 1. *Composition of basal medium*

Component	Weight/ml. of basal medium
Acid-hydrolyzed casein	10 mg.
dl-Tryptophan	0·1 mg.
l-Cystine	0·2 mg.
Glucose	20 mg.
Sodium acetate	20 mg.
Xylose	1 mg.
Calcium pantothenate	0·1 μg.
Pyridoxin	0·1 μg.
Riboflavin	0·2 μg.
p-Aminobenzoic acid	0·1 μg.
Biotin	0·0004 μg.
Adenine	0·01 mg.
Guanine	0·01 mg.
Uracil	0·01 mg.
Xanthine	0·01 mg.
Sodium chloride	5 mg.
Inorganic salts—solution A	See text
Inorganic salts—solution B	

Preparation of stock solutions

The stock solutions described below are preserved in the presence of a thin layer of toluene and stored in a refrigerator. All stock solutions and extracts are prepared with glass-distilled water. Possible contamination of any of the constituents of the medium with nicotinic acid was always tested for by using *Proteus vulgaris* in Stephenson's (1938) medium of known composition with sodium lactate as carbon source.

Acid-hydrolyzed casein. The various 'vitamin-free' caseins on the market all contain sufficient nicotinic acid to give high blanks with *L. arabinosus*. The following method of preparation of the hydrolysate consistently gave low blanks. 100 g. of a 'vitamin-free' casein are shaken twice for 20 min. with 300 ml. of 85% ethanol and filtered. The casein is

then added gradually to distilled water (1 l.) at 50° with constant stirring. The mixture is stirred for 30 min. and a 30% NaOH solution is slowly added, still with constant stirring, until the casein passes into solution. The viscous mass is stirred for 20 min., the mixture then adjusted to pH 4·6 with 10% HCl, the precipitate allowed to settle, and the whole then filtered. This operation is repeated three or four times. The alkali-extracted casein is then well washed with water at 50° and hydrolyzed with 500 ml. of 25% w/v H_2SO_4 for 10 hr. at 15 lb. pressure in an autoclave, or refluxed for 16–20 hr. on a sand-bath. 200 g. of litharge are added to the hydrolysate and the precipitate of lead sulphate filtered off. Barium hydroxide is now added to neutralize the remaining sulphuric acid and the barium sulphate removed by filtration. The hydrolysate is adjusted to pH 3, the volume made up to 700 ml., shaken for 30 min. with an active charcoal, e.g. Norite A, and filtered. The pH is adjusted to 6·8 with barium hydroxide. The resulting hydrolysate should be almost colourless. The solution is adjusted by evaporation or dilution to contain 100 mg./ml. of dry matter and is preserved under toluene in a refrigerator. It is usual after a few days for tyrosine to precipitate; this can be disregarded.

dl-*Tryptophan.* Natural *l*-tryptophan was so variable in its behaviour, owing to contamination with nicotinic acid, that its use was abandoned and the synthetic product prepared by Glaxo Laboratories, Ltd. was always used. *dl*-Tryptophan (2·0 g.) is suspended in approximately 500 ml. of hot water and concentrated HCl added drop by drop until solution is effected. The solution is then made up to a final volume of 1000 ml.

l-*Cystine.* *l*-Cystine (4·0 g.) is suspended in 500 ml. of hot water, 5 ml. of concentrated HCl added, and the solution made up to a final volume of 1000 ml.

Glucose, sodium acetate, xylose and *sodium chloride* are weighed out as required. Hydrated sodium acetate was used in preference to the anhydrous salt (1·66 g. of $CH_3.COONa.3H_2O \equiv 1$ g. CH_3COONa).

Biotin. An ampoule containing 25 μg. of the free acid is made up to 250 ml. with water. 1 ml. of inorganic salt solution A (see below) is also added before the solution is made up to volume. The solution thus contains 0·1 μg./ml. of biotin. *L. arabinosus* is insensitive to methyl biotin. If the methyl ester only be available it must be hydrolyzed to the free acid. The contents of an ampoule containing 25 μg. of the ester is added to 0·50 ml. of 0·1 N-HCl and the whole hydrolyzed for 1 hr. at 15 lb. pressure. 1 ml. of salt solution A is added and the pH adjusted to 6·8 with NaOH solution and the volume made up to 236 ml.

p-*Aminobenzoic acid.* *p*-Aminobenzoic acid (0·1 g.) is dissolved in 100 ml. of water containing two or three drops of glacial acetic acid. Before use this solution is diluted to one in ten so that each ml. contains 100 μg. The addition of *p*-aminobenzoic acid to the medium was first recommended by Isbell (1942), as this substance is removed from the casein hydrolysate by active charcoal. It has since been found (cf. Shankman, 1943) that there are two different types of strain of *L. arabinosus*, one of which requires *p*-aminobenzoic acid, while the other does not. In this investigation *p*-aminobenzoic acid was found to have a slight stimulating effect and was always added. It is recommended that the concentration of the acid in the final medium be not increased beyond that given here (0·1 μg./ml.); higher amounts, e.g. 1 μg./ml., have a definite depressing effect at the higher levels of nicotinic acid used for the standard curve.

Aneurin, calcium pantothenate and pyridoxin. A stock solution of these substances is prepared by dissolving 0·1 g. of each in 100 ml. of water. The stock solution is diluted one in ten before use.

Standard nicotinic acid. Pure nicotinic acid (0·1 g.) is dissolved in 100 ml. of water. This solution is diluted to give a standard solution containing 0·1 μg./ml.

Specificity. Nicotinic acid is a specific growth factor for *L. arabinosus*. Runs were set up with picolinic acid (pyridine-2-carboxylic acid), isonicotinic acid (pyridine-4-carboxylic acid) and quinolinic acid (pyridine-2:3-dicarboxylic acid) and no growth was obtained even when the concentration of these substances was raised to 1 μg./10 ml. of medium.

Riboflavin. A solution of riboflavin is prepared to contain 100 μg./ml. in 0·02 N-acetic acid.

Adenine, guanine and uracil. The stock solution of these three substances contains 1 mg./ml. Solution is effected by prolonged heating in the presence of a few drops of concentrated hydrochloric acid.

Xanthine. This solution also contains 1 mg./ml. Solution is effected by the addition of a few drops of strong ammonia.

The various stock solutions of vitamins, with the exception of biotin, are renewed at weekly intervals and the adenine, guanine, uracil and xanthine solutions are renewed at fortnightly intervals.

Inorganic salts, solution A. This is composed of 25 g. of K_2HPO_4 and 25 g. of KH_2PO_4 in 250 ml. of water.

Inorganic salt, solution B. This solution was modified to contain 10 g. $MgSO_4.7H_2O$, 0·5 g. $MnSO_4.4H_2O$ and 0·03 g. $FeCl_3$ in 250 ml. of water. Five drops of concentrated hydrochloric acid are added to the mixture.

Preparation of inoculum

A heavy growth of inoculum is required for a successful assay by this method. The best results were obtained when a transfer was made from the agar stab culture to a tube of the Snell & Strong riboflavin medium (see Barton-Wright & Booth, 1943). This is incubated for 18–20 hr. at 37°, centrifuged aseptically and resuspended in twice the volume of 0·9% saline solution. Particularly heavy growth is obtained in this medium.

The fermentations are carried out in ordinary chemical or bacteriological tubes (18 × 150 mm.) and the acid produced is titrated against 0·1 N-NaOH, with bromothymol blue as indicator. The titrations are carried out as described by Barton-Wright & Booth (1943).

Preparation of extracts

Dry materials are finely ground and 5 g. amounts are suspended in 50 ml. of N-HCl and autoclaved for 15 min. at 15 lb. pressure. Although *L. arabinosus*, unlike *L. helveticus*, is not stimulated by the presence of fats, materials which contain a high proportion of fat, e.g. wheat germ, maize, oats, soybean, meat, etc., should nevertheless be defatted by a preliminary Soxhlet extraction with light petroleum for 10 hr. This procedure prevents the formation of oily emulsions which may hinder complete extraction. After cooling, the extract is adjusted to pH 6·8 with 30% NaOH and the volume is made up to 100 ml. The concentration of the final solution of the extract should contain as nearly as possible 0·05 μg./ml. of nicotinic acid. This is effected by pipetting the necessary volume of sample from the initial 100 ml. of solution into distilled water and making up the volume to 500 ml. This solution is preserved under toluene.

Assay procedure

The following amounts of the stock solutions will give sufficient medium for 100 tubes:

Acid-hydrolyzed casein	100 ml.
l-Cystine solution	50 ml.
dl-Tryptophan solution	50 ml.
Anhydrous glucose	20 g.
Anhydrous sodium acetate	20 g.
Xylose	1·0 g.
Sodium chloride	5·0 g.
Biotin solution	4·0 ml.
p-Aminobenzoic acid solution	1·0 ml.
Aneurin, calcium pantothenate and pyridoxine solution	1·0 ml.
Riboflavin solution	2·0 ml.
Adenine + guanine + uracil solution	10·0 ml.
Xanthine solution	10·0 ml.
Inorganic salt solution A	5·0 ml.
Inorganic salt solution B	5·0 ml.

After mixing, the medium is adjusted to pH 6·8 with sodium hydroxide and the volume made up to 500 ml. with water. This gives a mixture having twice the concentration of the final assay medium. 5 ml. of the medium are transferred to each tube. Sixteen tubes are kept for the blanks and the determination of the standard nicotinic acid curve. To the series of tubes are added serially 5 ml. of water (= blank), 0·5, 1·0, 1·5, 2·0, 2·5, 3·0, 3·5 ml. of standard nicotinic acid solution (0·1 μg./ml.). In each case sufficient distilled water is added to bring the final volume of each tube to 10 ml. The remaining tubes are used for the assay of the extracts.

It is necessary to carry out each assay at four different levels. It will be found convenient to take 1·0, 2·0, 3·0, 4·0 ml. of each extract for an assay. All concentration levels of standard nicotinic acid solution and extracts are set up in duplicate. After the volume of the tubes has been adjusted to 10 ml. they are plugged with cotton-wool and autoclaved for 10 min. at 10 lb. pressure, cooled and inoculated with one drop of inoculum per tube from a sterile pipette. The tubes are incubated for 72 hr. at 37° and then titrated. Good results can be obtained after 48 hr. incubation, but it was found to be more convenient in routine work to maintain a standard incubation time of 72 hr.

The standard curve (Fig. 1) is plotted in the usual way and the nicotinic acid content of each concentration level of an extract determined from it. The mean of at least three readings, not differing among themselves by more than $\pm 10\%$, should be taken.

ASSAY RESULTS

A wide range of materials was assayed for nicotinic acid, including different varieties of wheat, various fractions of the wheat grain, wheaten flours, other cereals, e.g. maize, rye, oats and barley, as well as yeast, dried meat, ordinary and dried milk, beer, vinegar, coffee, cocoa and tea. The results of a number of different determinations on various cereals and preparations therefrom are given in Tables 2–7; the results are calculated on the normal water contents of the products.

A survey of the nicotinic acid content of the milling fractions of five different Manitoba wheats specially milled by my colleague Mr A. G. Simpson for this work gave the results shown in Table 4.

Table 2. *Nicotinic acid content of wheat and wheat grain fractions*

	Sample	Nicotinic acid content (μg./g.)
1	Wheat, English	48·0
2	Wheat, Manitoba	55·0–66·0
3	Wheat, germ, commercial sample 1	55·0
4	Wheat, germ, commercial sample 2	75·0
5	Wheat, germ, commercial sample 3	77·0
6	Wheat, bran	267–325
7	Flour, National (85% extraction)	12·0–18·7

Table 3. *Nicotinic acid content of wheat flour fractions*

	Sample English wheat (%)	Nicotinic acid content (μg./g.)
Extraction	45	5·0
	70	7·5
	75	8·0
	80	9·0
	85	10·5
	100	48·0
	Manitoba wheat (%)	
Extraction	40	7·0
	70	8·4
	75	9·6
	80	11·0
	85	13·3
	100	55·0

A number of beers and vinegars was also assayed for nicotinic acid content. The results are shown in Table 8. It is of interest to compare the nicotinic acid content of modern beers, samples 1–7, with those brewed 45, 70 and in one case 150 years ago. The specimen called 'Chancellor' is abnormally high in nicotinic acid. The same is true of its riboflavin content (3·9 μg./g.).

The opportunity was taken to assay the nicotinic acid of various hopped and unhopped worts and to compare the values with the gravities of the liquors (Table 9).

It will be seen from Table 9 that there is a direct correlation between the nicotinic acid content and the gravity of the liquors. The values for miscellaneous products assayed for nicotinic acid are given in Table 10.

DISCUSSION

Lactobacillus arabinosus is less exacting than *L. helveticus* (*L. casei* ϵ) in its vitamin requirements. Only three growth-factors need be given for normal growth, namely nicotinic acid, pantothenic acid and biotin, whereas *L. helveticus* requires in addition riboflavin, pyridoxin (or 'pseudopyridoxin') and the at present ill-defined factor or factors contained in 'folic acid' concentrates, which are to be found

Table 4. *Nicotinic acid content of different milling fractions of Manitoba wheat*

Sample	%	Nicotinic acid content (μg./g.)	Product
A. No. 1 Northern Manitoba export standard, 1943 crop			
Grist	100	62·5	6250
Flour	74·5	13·5	1006
Middlings*	2·9	100·0	290
Fine bran†	10·0	120·0	1200
Coarse bran‡	12·6	298·0	3755
Total			6251
Difference			+1
B. No. 2 Northern Manitoba export standard, 1943 crop			
Grist	100	62·5	6250
Flour	75·1	12·8	961
Middlings*	2·4	105·0	252
Fine bran†	10·0	142·0	1420
Coarse bran‡	12·5	281·0	3510
Total			6143
Difference			−107
C. No. 3 Northern Manitoba export standard, 1943 crop			
Grist	100	62·8	6280
Flour	73·2	13·6	995
Middlings*	3·5	97·5	340
Fine bran†	11·4	150·0	1710
Coarse bran‡	12·0	267·0	3210
Total			6255
Difference			−25
D. No. 4 Northern Manitoba export standard, 1943 crop			
Grist	100	62·5	6650
Flour	72·2	11·5	834
Middlings*	3·8	81·0	308
Fine bran†	11·8	145·0	1710
Coarse bran‡	12·2	325·0	3910
Total			6762
Difference			+112
E. No. 5 Wheat (Manitoba) export standard, 1943 crop			
Grist	100	66·0	6600
Flour	68·7	14·0	962
Middlings*	5·7	75·0	427
Fine bran†	13·3	131·0	1740
Coarse bran‡	12·35	285·0	3520
Total			6649
Difference			+49

* 'Middlings' contains all the remaining residual endosperm and also some germ.

† 'Fine bran' contains a little more endosperm and a fair proportion of the germ of the wheat.

‡ 'Coarse bran' is defined as the outer coat of the grain practically free from endosperm.

in peptone, liver, kidney and yeast. In the present investigation several runs were made in which aneurin, riboflavin and pyridoxin were omitted from the medium. In agreement with previous work (cf. Bohonos, Hutchings & Peterson, 1941; Snell & Wright, 1941; and Krehl *et al.* 1943), perfectly satisfactory results were obtained. It is therefore possible that these factors are unnecessary. Nevertheless, it was considered undesirable, in all the circumstances, to omit these substances at this stage of the investigation.

Table 5. *Nicotinic acid content of barleys and malts*

Sample	Nicotinic acid content (μg./g.)
1. Barley. Unnamed commercial variety	91·5
2. Barley. Block	85·0
3. Barley. Irish Archer	147·0
4. Barley. Plumage	124·0
5. Barley. Arch Plume	120·0
6. Barley. Spratt	131·0
7. Barley. Archer Goldthorpe	120·0
8. Barley. Spratt Archer	120·0
9. Barley. Irish Goldthorpe	120·0
10. Commercial malt. Sample 1	100·0
11. Commercial malt. Sample 2	108·0
12. Commercial malt. Sample 3	108·0
13. Commercial malt. Sample 4	91·0

(Samples 3–9 were unhusked grains.)

Table 6. *Nicotinic acid content of starchy and sweet maizes*

Sample	Nicotinic acid content (μg./g.)
A. Starchy maize	
1. Manalta	11·5
2. Beacon	18·0
3. Howes Alberta	11·5
4. Jehu	20·0
5. B 45	18·8
6. Quebec 28	15·0
7. Mixed seed variety trial	14·2
Mean	15·6
B. Sweet maize	
8. John Innes hybrid no. 1	28·0
9. John Innes hybrid no. 2	33·0
10. Canada Gold	37·0
11. C 13	26·0
Mean	31·3

Table 7. *Nicotinic acid content of rye and oat varieties*

Sample	Nicotinic acid content (μg./g.)
Oats, Victory	6·4
Oats, Monarch	6·5
Oats, Star Seed	6·3
Oats, Royal Scot	6·5
Oats, Golden Rain	5·5
Oats, Ayr Commando	4·9
Oats, Ayr Bounty	6·8
Oats, Bell	7·0
Rye, Carter's Giant Winter	10·0
Rye, unnamed variety	9·3
Rye, unnamed variety	7·7

According to Brown, Thomas & Bina (1943), oxidation with hydrogen peroxide destroys approximately 50 % of the nicotinic acid content of wheat.

Table 8. *Nicotinic acid content of beers and vinegars*

Sample	Nicotinic acid content (μg./g.)
1. Modern commercial samples	7·8
2. Modern commercial samples	8·2
3. Modern commercial samples	9·5
4. Modern commercial samples	12·0
5. Modern commercial samples	12·5
6. Modern commercial samples	14·4
7. Modern commercial samples	17·0
8. 'Chancellor'. (All Souls College, Oxford, brewed 1938)	45·6
9. Worthington Strong Ale (brewed 1798)	16·5
10. Anglo-Bavarian Beer (brewed 1872)	10·3
11. Reid's Stout (brewed 1899)	16·0
12. Combes Export Brown Stout (brewed 1900)	15·3
13. Stock Vinegar	9·5
14. Tarragon Vinegar	9·0

Extraction of cereal samples with water alone will not remove the whole of the nicotinic acid content of the grain. There is also present in cereals a nicotinic acid precursor (cf. Melnick, 1942), which is converted to nicotinic acid in the presence of alkali in the cold, but with acid only on heating. It is probable that Brown *et al.* failed to extract the whole of the nicotinic acid from their samples, since they used water alone for this purpose.

The low nicotinic acid content of rye and oats is surprising. It might have been expected that the values would have been of the same order as that of wheat and barley. In the case of maize the nicotinic acid content of starchy maize is approximately 50 % of that of sweet varieties. It has been known for some time that the nicotinic acid content of maize is low and maize is said to be a component of a pellagra-producing diet (maize, salt meat and molasses). Nevertheless, compared with rye and oats the nicotinic acid content of maize is relatively high and it would be of interest to know whether the incidence of pellagra in rye-consuming countries, e.g. Italy and the Balkans, is connected with the low nicotinic acid content of this cereal.

Table 9. *Nicotinic acid content and gravity of hopped and unhopped worts*

Sample	Nicotinic acid content (μg./ml.)	Gravity
1. *Unhopped wort.* Samples drawn at intervals and mixed to represent liquors drained off from the mash tun at various stages	19·5	1096·58
2.	15·1	1071·46
3.	6·5	1014·50
4. Mixture of above representing the whole extract used in the brew	10·5	1045·71
5. *Hopped wort.* Mixture of different strengths of wort samples above but *after* boiling with hops and before adding yeast	11·0	1045·85
6. Beer	8·2	1034·0

Table 10. *Miscellaneous products*

Sample	Nicotinic acid content (μg./g.)
1. Dehydrated meat	100–110
2. Yeast extract 1 (*Torula utilis*)	2077
3. Yeast extract 2	655
4. Dried brewer's yeast	600
5. Yeast extract 4	275–288
6. Coffee	132
7. Cocoa	16
8. Tea	61
9. Milk powder	9·2
10. Milk	0·8–1·0 (μg./ml.)

Krehl *et al.* (1943) were unable to confirm this statement. A repetition of this work in the present investigation also failed to confirm this suggestion.

SUMMARY

1. A number of modifications and improvements have been made in the original Snell & Wright medium for the microbiological assay of nicotinic acid, using *Lactobacillus arabinosus* 17/5.

2. The nicotinic acid content of cereals, cereal products and other foods has been assayed.

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Mono-iodotyrosine

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3:5-Di-iodotyrosine has been known for many years as a natural product, having been isolated from natural iodine-containing proteins such as gorgonin (Drechsel, 1895) and thyroglobulin (Harington & Randall, 1929); the process of isolation has usually involved alkaline hydrolysis so that the di-iodotyrosine obtained has been racemized, but the optically active compound has also been isolated by enzymic hydrolysis of thyroglobulin (Harington & Randall, 1931). The *dl*-compound has further been obtained by alkaline hydrolysis of artificially iodinated proteins (Oswald, 1911; Ludwig & von Mutzenbecher, 1939). Both the natural optically active di-iodotyrosine and the *dl*-amino-acid have been prepared synthetically by iodination of *l*- and *dl*-tyrosine respectively.

In contrast with this extensive information relating to di-iodotyrosine, references to mono-iodotyrosine have only recently appeared in the literature. In the course of their work on iodinated proteins Ludwig & von Mutzenbecher (1939) describe the isolation of mono-iodotyrosine from iodinated casein. The compound was obtained from the mother liquors of the fraction of the products of alkaline hydrolysis which contained di-iodotyrosine, after separation of the latter; its constitution was established by elementary analysis and by the facts that it gave 3:5-di-iodotyrosine on treatment with 1 mol. of iodine in alkaline solution and tyrosine on catalytic de-iodination.

More recently Herriott (1941–2) has given an account of the isolation of mono-iodotyrosine from partially iodinated crystalline pepsin. Herriott's description of the compound differs in several respects from that given by Ludwig & von Mutzenbecher. Thus his preparation separates from water in diamond-shaped crystals which are anhydrous, whilst that of the German authors is described as forming whetstone-shaped needles with $1H_2O$; in both cases the compound is stated to give the colour reaction with nitrous acid and ammonia which is characteristic of thyroxine and di-iodotyrosine, and Herriott states that the Millon reaction is also positive. Ludwig & von Mutzenbecher make no mention of optical activity; Herriott claims that his compound has $[\alpha]_D^{22°} - 8{\cdot}8°$, an observation which is very surprising since the method of isolation from iodinated pepsin involves prolonged hydrolysis with concentrated barium hydroxide; such treatment should lead to complete racemization of any tyrosine derivative. In any case, since the preparation of Ludwig & von Mutzenbecher was isolated from a similar hydrolysate the difference between it and that of Herriott cannot reasonably be ascribed to difference in stereochemical configuration.

During the working up of the mother liquors from an experiment on the oxidation of di-iodo-*l*-tyrosine (shortly to be described in another paper), in which not very highly purified di-iodotyrosine was employed as starting material, we ourselves encountered a crystalline amino-acid which analyzed for mono-iodotyrosine but did not agree entirely in its properties with either of the compounds described by Herriott and by Ludwig and von Mutzenbecher. Since we were anxious to establish the identity of our product without doubt we decided to attempt the synthesis of mono-iodo-*l*- and *dl*-tyrosine by unequivocal methods. This we have accomplished by conversion of *l*- and *dl*-tyrosine respectively into the corresponding 3-amino derivatives (Waser & Lewendowski, 1921), diazotization of the latter and decomposition of the diazonium salts with iodide.

3-Iodo-*l*-tyrosine obtained in this manner crystallizes in blunt needles or lozenge-shaped crystals which are anhydrous; in N-hydrochloric acid it has $[\alpha]_D^{20°} - 4{\cdot}4°$; in both these respects it is identical with the compound isolated by us as a by-product from impure di-iodo-*l*-tyrosine and is different from the preparations both of Herriott and of Ludwig & von Mutzenbecher.

Synthetic 3-iodo-*dl*-tyrosine on the other hand forms triangular or diamond-shaped plates of which the corners are frequently rounded so that they appear with the outline of a whetstone; the crystals contain $1H_2O$ which cannot be removed without decomposition and loss of iodine. The compound thus corresponds closely with that described by Ludwig & von Mutzenbecher.

We therefore conclude that the compound isolated by the latter authors was unquestionably 3-iodo-*dl*-tyrosine; on the other hand we have found nothing resembling the substance described by Herriott and we are unable to offer any explanation of his results.

EXPERIMENTAL

A. 3-*Iodo*-l-*tyrosine*

3-Amino-*l*-tyrosine (Waser & Lewandowski, 1921) (6 g.) was dissolved in cold H_2SO_4 (45 ml. of 1:5); the solution was diluted with water (60 ml.), cooled to $-2°$ and diazotized by addition of a solution of $Ba(NO_2)_2$ (3·51 g.) in water (60 ml.), the temperature being maintained between 0° and $-2°$. The solution was then poured into boiling concentrated aqueous KI (25 g. : 5 equiv.) containing copper bronze in suspension; the addition was made at such a rate that ebullition was not interrupted, instantaneous decomposition of the diazonium salt being essential for good results.

The mixture was filtered hot and the filtrate evaporated under diminished pressure; on taking up the residue in hot water and cooling, a first crop of 3·4 g. was obtained; a second crop, contaminated with salt, was isolated by concentration of the mother liquor. Recrystallization of the united crops from water (80 ml.; charcoal) gave 4·4 g. (47% of theory) of pure material.

The pure 3-iodo-*l*-tyrosine dissolved in about 15 parts of boiling water from which it crystallized in blunt needles or lozenge-shaped crystals. It had m.p. 204–206° (decomp.). (Found: C, 35·6; H, 3·8; N, 4·8; I, 41·1%. $C_9H_{10}O_3NI$ requires C, 36·1; H, 3·7; N, 4·6; I, 41·4%.) In N-HCl the amino-acid had $[\alpha]_D^{20°} - 4·4°$ ($c = 5$).

In order to ensure that no racemization had occurred during the synthesis, part of the 3-iodotyrosine was converted into tyrosine by shaking in alkaline solution in an atmosphere of hydrogen with palladized strontium carbonate as catalyst; 1 g. of 3-iodo-*l*-tyrosine ($[\alpha]_D^{20°} - 4·4°$) gave 0·56 g. (95% of theory) of tyrosine having $[\alpha]_D^{20°} - 12·4°$ ($c = 5$ in N-HCl).

B. 3-*Iodo*-dl-*tyrosine*

3-*Nitro*-dl-*tyrosine*. *dl*-Tyrosine (8 g.) was suspended in water (30 ml.) and treated with HNO_3 (21·4 ml. of sp. gr. 1·42), the temperature being kept below 25°. The nitrate of the product crystallized rather slowly; after being kept for some time at 0° it was collected and suspended in a small amount of water; ammonia (sp. gr. 0·880) was added until the suspension was neutral and the whole was brought to the boil, more ammonia being added as necessary to maintain neutrality to Congo Red. After cooling, the amino-acid was collected, washed and dried. Yield, 7·25 g. (72·5% of theory).

For analysis it was recrystallized by boiling the solution in 0·1 N-HCl with charcoal and neutralizing the hot filtrate with ammonia; it separated in yellow prismatic needles, m.p. 214–215° (decomp.). (Found: N (Dumas), 12·1%; $C_9H_{10}O_5N_2$ requires N, 12·4%.)

3-*Amino*-dl-*tyrosine*. This was prepared from 3-nitro-*dl*-tyrosine precisely as described by Waser & Lewandowski (1921) for the *l*-isomeride; 7·25 g. of the nitro compound gave 3·02 g. (48% of theory) of 3-amino-*dl*-tyrosine, crystallizing from water in colourless stumpy prisms having m.p. 288° (decomp.). (Found: N, 14·5%; $C_9H_{12}O_3N_2$ requires N, 14·3%.)

3-*Iodo*-dl-*tyrosine*. The diazotization and decomposition of the diazonium salt were carried out as described above for the *l*-isomeride; the yield of re-crystallized 3-iodo-*dl*-tyrosine was 44% of theory. The amino-acid crystallized from water in triangular or diamond-shaped plates, often with rounded corners, containing $1H_2O$ and having m.p. 200–201° (decomp.). The solubility was similar to that of the *l*-isomeride. (Found: C, 33·7; H, 3·9; N, 4·4; I, 38·8%. $C_9H_{10}O_3NI.H_2O$ requires C, 33·2; H, 3·7; N, 4·3; I, 39·1%.)

Colour reactions of mono-iodotyrosine

In contrast with di-iodotyrosine, mono-iodotyrosine gives the Millon reaction with intensity. On the other hand, we are not able to agree with Ludwig & von Mutzenbecher or with Herriott that it gives the colour reaction with nitrous acid and ammonia which is characteristic of thyroxine and which is also given, though with less intensity, by di-iodotyrosine. If 3-iodotyrosine is boiled with aqueous-ethanolic HCl and a large excess of nitrite, an orange-red colour is indeed obtained on addition of excess of ammonia; under the conditions commonly employed for the test, however, no trace of red colour is to be observed.

SUMMARY

1. 3-Iodo-*l*-tyrosine and 3-iodo-*dl*-tyrosine have been synthesized, and their properties have been compared with those of reputed preparations of mono-iodotyrosine, accounts of which have recently appeared.

2. It is concluded that the compound obtained by Ludwig & von Mutzenbecher (1939) from iodinated casein was in fact 3-iodo-*dl*-tyrosine; that isolated by Herriott (1941–2) from iodinated pepsin does not correspond with either of the synthetic compounds and its nature remains in doubt.

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* after neutralization to pH 6-4 with concentrated ammonia,

The Isolation of a Δ^5-androstene-3 (β), 16, 17-triol from the Urine of Normal Human Males and Females

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In 1934 one of the authors isolated from the neutral ether-soluble fraction of acid-hydrolyzed human pregnancy urine a new solid alcohol melting at *c.* 263°. The alcoholic nature of the compound was demonstrated by the preparation of an acetate, but owing to the small amount available, little could be done to characterize it further. Subsequently, a systematic search for this new compound revealed its presence in small amounts in nearly all specimens of urine from normal men and normal non-pregnant women which were examined. Although the average yield from such urines was only about 0·1 mg./l., sufficient material was accumulated to permit a re-investigation of its nature.

The new substance was very sparingly soluble in benzene, ethyl acetate, light petroleum and chloroform; rather more soluble in acetone, methanol and ethanol, and still more soluble in dioxan and glacial acetic acid. The analytical specimen was obtained in fine white needles melting at 262–263° after one crystallization from methanol-benzene and two from methanol. Tests for halogen, nitrogen, sulphur and phosphorus were negative. The possible steroid nature of the substance was indicated by a positive Liebermann-Burchard reaction in which the chloroform layer showed a transient purple colour. Tests with tetranitromethane and with the Rosenmund-Kuhnhenn bromine reagent showed that it was unsaturated.

An acetate, m.p. 189–191°, a chloroacetate, m.p. 161–163°, a hydrogenation product, m.p. 257·5–259° and an acetate and chloroacetate of the latter melting at 174–176° and 180–182° respectively were prepared. Analyses of these derivatives gave figures, however, which could not be reconciled with any of the possible multiples of the empirical formula the analysis of the original compound seemed to indicate.

On treatment of the compound with lead tetra-acetate, an oxidation product, m.p. 199–201°, was obtained in small yield. This indicated that the compound might have two hydroxyl groups on adjacent carbon atoms, but again the analysis of the product could not be reconciled with any of the formulae which were then being considered for the original compound.

It was hoped that further amounts of the compound could be prepared so that a fuller investigation of its nature could be made. However, owing to other claims on the time of the authors during the past few years this has not been possible.

The reinvestigation of this compound now no longer seems to be necessary, since it appears to be identical with a steroid which has recently been isolated from the urine of a boy with an adeno-carcinoma of the adrenal cortex by Hirschmann (1943) and satisfactorily characterized as a Δ^5-androstene-3 (β), 16, 17-triol. In Table 1 the analytical figures and melting-points for the compound isolated by the present authors and those of certain of its derivatives are compared with the corresponding figures for Hirschmann's androstenetriol and its derivatives.

Table 1. *Comparison of the steroid isolated from the urines of normal men and women* (X) *with Hirschmann's* Δ^5*-androstene-3* (β), 16, 17*-triol*

	X			Δ^5-Androstene-3 (β), 16, 17-triol						
	m.p.	% C	% H		m.p.	% C	% H	Calc. for	% C	% H
				Androstenetriol	265–270°	74·54	9·87	$C_{19}H_{30}O_3$	74·47	9·87
X	262–263°	73·05	9·65	Androstenetriol from hydrolysis of acetate and monoacetate	—	72·72	9·95	$C_{19}H_{30}O_3 + \frac{1}{2}CH_3OH$	72·63	10·00
					268–271°	72·97	10·35			
					266–270°	72·78	10·06			
X acetate	189–191°	69·43	8·33	Androstenetriol tri-acetate	187–188·5°	69·23	8·43	$C_{25}H_{36}O_6$	69·42	8·39
Hydro-genated *X*	257·5–259°	72·44	10·20	Androstanetriol	256–260°	73·92	10·38	$C_{19}H_{32}O_3$	73·98	10·46
								$C_{19}H_{32}O_3 + \frac{1}{2}CH_3OH$	72·16	10·57
Hydro-genated *X* acetate	174–176°	68·82	9·15	Androstanetriol tri-acetate	175·5–176·5°	68·44	8·91	$C_{25}H_{38}O_6$	69·09	8·81
						68·99	8·27			

The melting-points agree so closely in the case of the acetate, the hydrogenation product, and the acetate of the latter, that the identity of the two series of compounds with one another can hardly be doubted. Furthermore the melting-points of the acetate of the compound and of the acetate of its hydrogenation product were not depressed by admixture with androstenetriol triacetate and androstanetriol triacetate respectively, specimens of which were most kindly supplied by Dr Hirschmann. The melting-point recorded for the androstenetriol itself is, however, appreciably higher than any observed by the present authors for their product, which would seem to indicate that the latter was never obtained in a state of complete purity. This is hardly surprising since at no time were large enough amounts of the substance on hand to allow of a really rigorous purification.

The carbon and hydrogen contents of the acetate and of the acetate of the hydrogenated substance agree excellently with the theoretical figures for the acetates of an androstenetriol and of an androstanetriol respectively. In the cases of the original steroid and its hydrogenation product, the agreement with the theoretical figures for an androstenetriol and an androstanetriol is less satisfactory. These discrepancies, however, can be explained in a not unreasonable manner. By saponification of the diacetate and monoacetate of the triol, Hirschmann obtained products which showed the same melting-point as that of the original substance and whose identity with the latter was clearly shown by mixed melting-points and by reacetylation to the triacetate. However, these products gave analytical figures which suggested that half a molecule of methanol of crystallization had been retained even though they were dried *in vacuo* at temperatures as high as 135°. While it was not made clear why the androstenetriol prepared by saponification of the acetates should retain methanol of crystallization while that isolated directly from urine did not, Hirschmann's figures suggest that this was the case with the particular samples which were analyzed. It seems reasonable to assume, therefore, that the discrepancies in the carbon and hydrogen figures observed by the present authors, which are referred to above, might be explained in a similar manner by supposing that methanol of crystallization was retained by the substance itself and by its hydrogenation product.

Hirschmann has raised the interesting possibility that this androstenetriol may be formed from dehydro*iso*androsterone by a metabolic reaction similar to that by which oestrone is converted to oestriol in the body (Pearlman & Pincus, 1943). The demonstration that the same androstenetriol is present in the urines of normal men and women, in both of which dehydro*iso*androsterone is known to occur (Butenandt & Tscherning, 1934; Callow & Callow, 1939), lends indirect support to the theory that the latter may be the precursor of the triol. What is more important, however, is that it is now clearly shown that the metabolic path by which the androstenetriol is formed is a normal one.

EXPERIMENTAL

Isolation of Δ^5-androstene-3 (β), 16, 17-triol from urine. The urine was collected in toluene, evaporated under reduced pressure to one-tenth of its original volume, and after acidification to pH 1·5 with HCl it was heated for 2 hr. in a boiling water-bath. The concentrated hydrolyzed urine was extracted four times with one-quarter volumes of ether, and the ethereal extract after washing thoroughly with 5% Na_2CO_3 solution, 0·1 N-NaOH solution, and water, was evaporated to dryness.

The subsequent steps by which the crude triol was isolated from the neutral ether-soluble fraction and by which it was purified were varied considerably from batch to batch, depending on the amount of triol present and the degree of contamination with impurities. In some cases good yields of crude material were deposited on chilling a benzene solution of the neutral fraction. It was found in other cases to be advantageous to give the neutral fraction a preliminary leaching with light petroleum to remove waxy impurities which seemed to interfere with the crystallization from benzene. A preliminary saponification of the neutral fraction with 5% ethanolic KOH and subsequent ether extraction of the non-saponifiable material was also found to be useful as a preliminary to the benzene crystallization. With certain batches of urine good yields of crude crystalline triol were obtained by dissolving the neutral fraction in a small volume of ethyl acetate, adding light petroleum until a faint cloudiness appeared, and then chilling for several days.

Purification of the crude crystalline material obtained in these ways could usually be effected by washing with ice-cold chloroform and crystallizing from ethyl acetate-methanol or benzene-methanol. The melting-points of preparations purified by one such crystallization were usually in the range 255–260°. In some cases in which crystallization was difficult, the crude material was acetylated, and the crude acetates sublimed at 165° in a high vacuum and subsequently saponified. The material so obtained was found to crystallize readily from ethyl acetate-methanol or benzene-methanol.

In Table 2 are shown the yields and melting-points of the once-crystallized material obtained from the urines of five non-pregnant women and of four men. Further quantities were obtained for chemical examination from large volumes of mixed urines from men.

A sample for analysis, m.p. 262–263°, was obtained by recrystallizing the combined crystals from several batches once from benzene-methanol, and twice from methanol.

4·490 mg. (dried at 80° *in vacuo* over P_2O_5) gave 12·020 mg. CO_2 and 3·870 mg. H_2O. (Found: C, 73·05; H, 9·65%. Calc. for $C_{19}H_{30}O_3 . \frac{1}{2}CH_3OH$: C, 72·63; H, 10·00%.)

$[\alpha]_{5461}^{22.5°} = -88·5°$ (0·56% in dioxan); $[\alpha]_{5461}^{26°} = -75·7°$ (0·63% in 90% acetic acid).

Determinations of the iodine value by the method of Rosenmund & Kuhnhenn on 4·027 and 4·701 mg. gave values of 57·3 and 65·8 respectively. The calculated iodine value for $C_{19}H_{30}O_3 . \frac{1}{2}CH_3OH$ (1°), is 78·6.

Table 2. *Yields of once-crystallized androstenetriol from urines of non-pregnant women and of men*

	Vol. of urine (l.)	Yield of crystals (mg.)	m.p. of crystals
Female	96·5	60·0	255–260°
	149·8	45·5	258–261°
	52·9	9·6	252–258°
	20·3	16·6	255–262°
	31·3	4·4	259–263°
Male	54·3	6·5	258–261°
	53·0	3·4	260–262°
	49·6	3·0	258–260°
	48·4	7·0	255–260°

Δ^5-*Androstene*-3-(β), 16, 17-*triol triacetate*. 20 mg. androstenetriol (m.p. 258–260°) were heated at 100° for 2 hr. with 2 ml. of acetic anhydride and one drop of pyridine. After decomposition of the excess acetic anhydride with ice and water, the crude acetate was filtered off, washed, dried, and crystallized from ether-hexane. Hexagonal plates, m.p. 189–191°, were obtained.

3·621 mg. (dried at 80° *in vacuo* over P_2O_5) gave 9·200 mg. CO_2 and 2·690 mg. H_2O. (Found: C, 69·43; H, 8·33%. Calc. for $C_{25}H_{36}O_6$: C, 69·42; H, 8·39%.)

Δ^5-*Androstene*-3 (β), 16, 17-*triol tri*(*chloroacetate*). 19·7 mg. of androstenetriol (m.p. 260–263°) were treated at 100° for 2 hr. with 4 ml. of chloracetyl chloride. The excess chloracetyl chloride was decomposed with cold water and the crude tri(chloroacetate) filtered off, washed with water and dried. Three crystallizations from 75% aqueous methanol yielded plates melting at 161–163°.

4·545 mg. (dried at 80° *in vacuo* over P_2O_5) gave 9·300 mg. CO_2 and 2·530 mg. H_2O. (Found: C, 55·83; H, 6·23%. Calc. for $C_{25}H_{33}O_6Cl_3$: C, 56·52; H, 6·14%.)

Δ^5-*Androstane*-3 (β), 16, 17-*triol*. Hydrogenation of the androstenetriol was carried out in glacial acetic acid solution with electrolytic hydrogen and a platinum oxide catalyst. Five experiments on batches of weight varying between 10 and 17 mg. were carried out. In every case the measured uptake of hydrogen was about 150% of that required for the saturation of a single double bond in a substance of the formula $C_{19}H_{30}O_3 \cdot \frac{1}{2}CH_3OH$. Little significance is attached to this discrepancy, however, since the hydrogenation apparatus used was not well suited for accurate quantitative work.

After removal of the catalyst by filtration, the solution of hydrogenated material was evaporated to dryness under reduced pressure and the residue crystallized from benzene-methanol. After three crystallizations crystals melting sharply at 257·5–259° were obtained. An iodine value determination showed that the product was saturated.

3·556 mg. (dried at 80° *in vacuo* over P_2O_5) gave 9·445 mg. CO_2 and 3·240 mg. H_2O. (Found: C, 72·44; H, 10·20%. Calc. for $C_{19}H_{32}O_3 \cdot \frac{1}{2}CH_3OH$: C, 72·16; H, 10·57%.)

$[\alpha]_{5461}^{25°} = 0·0°$ (0·50 and 0·52% in methanol).

The melting-point of the androstanetriol after storage for long periods in the dry state became lower and less sharp. When such material was wetted and rapidly redried, the melting-point returned to its original value. This peculiar change in the melting-point after long storage could not be duplicated by drying at 80° in a high vacuum over P_2O_5, sublimation at 155° and 0·05 mm. or by heating at 100° for 12 hr. in the presence of oxygen.

Δ^5-*Androstane*-3 (β), 16, 17-*triol triacetate*. 13·2 mg. of the androstanetriol were acetylated in the manner previously described. The product, after crystallization once from 50% aqueous methanol and once from 50% aqueous acetone, melted at 174–176°.

3·057 mg. (dried at 80° *in vacuo* over P_2O_5) gave 7·715 mg. CO_2 and 2·500 mg. H_2O. (Found: C, 68·82; H, 9·15%. Calc. for $C_{25}H_{38}O_6$: C, 68·44; H, 8·91%.)

Δ^5-*Androstane*-3 (β), 16, 17-*triol tri*(*chloroacetate*). A chloracetylation was carried out with 15 mg. of the androstanetriol in the same way as for the androstenetriol. The crude product crystallized from 75% aqueous methanol in plates melting at 180–182°.

2·057 mg. (dried at 80° *in vacuo* over P_2O_5) gave 4·150 mg. CO_2 and 1·130 mg. H_2O. 5·423 mg. (dried as before) gave 3·975 mg. AgCl. (Found: C, 55·05; H, 6·15; Cl, 18·12%. Calc. for $C_{25}H_{35}O_6Cl_3$: C, 56·31; H, 6·48; Cl, 19·57%.)

Oxidation of Δ^5-*androstene*-3 (β), 16, 17-*triol with lead tetra-acetate*. Two experiments were carried out in which 17·0 and 17·8 mg. amounts of somewhat impure androstenetriol were each allowed to react at room temperature for 20 hr. with 5 ml. of an 0·1 N solution of lead tetra-acetate in glacial acetic acid. The reaction mixtures, after heavy dilution with water, were extracted with ether, and the extracts, after thorough washing with 5% Na_2CO_3 solution and with water, were evaporated to dryness. The residues on sublimation at 160–170° and 0·1 mm. yielded in each case a mixture of a small quantity of white needles and a larger quantity of glassy material. A total of only 3·5 mg. of the needles (m.p. 199–201°) was obtained.

2·074 mg. (dried at 80° *in vacuo* over P_2O_5) gave 5·720 mg. CO_2 and 1·805 mg. H_2O. (Found: C, 75·21; H, 9·74%. Calc. for $C_{19}H_{28}O_3$: C, 74·95; H, 9·27%.)

SUMMARY

1. A substance isolated from the urines of normal men and women in amounts of about 0·1 mg./l. has been identified with the Δ^5-androstene-3 (β), 16, 17-triol which has been isolated in large amounts by Hirschmann from the urine of a boy with an adenocarcinoma of the adrenal cortex.

2. This finding indicates that the androstenetriol is a normal steroid metabolic product.

The authors wish to express their gratitude to their many colleagues who co-operated in the collection of urine, and to Dr Hirschmann who so promptly supplied specimens for mixed melting-point determinations. The analyses were carried out by Mrs Dorothy Jewitt.

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On Arginase and its Participation in Urea Synthesis in the Liver

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Since Bollman, Mann & Magath (1924) demonstrated that *in vivo* mammalian liver was the only site for the synthesis of urea, much effort has been directed to the investigation of the details of this synthesizing mechanism. The theory of the 'ornithine cycle' (Krebs & Henseleit, 1932) offered a plausible explanation, until Neber (1935), London & Alexandry (1937) and Leuthardt (1938), in similar experiments *in vivo* and *in vitro*, were unable to confirm the essential role attributed to ornithine in urea synthesis. Trowell more recently (1942), in perfusion experiments, failed to find more than a transitory effect on urea synthesis by the other members of the 'ornithine cycle', citrulline and arginine; he found, however, a catalytic action of ornithine under his experimental conditions. The experiments of Schoenheimer, Ratner & Rittenberg (1939) confirming the 'cycle' are not unambiguous. The workers after feeding with N^{15} found a high percentage of the isotope both in the urea and in the protein-linked arginine but not in the free arginine. They formulated the hypothesis that 'free arginine' and protein arginine are continuously exchanged in the liver, and that urea is formed from arginine while the latter is in the free state, for peptide-linked arginine is not hydrolyzed by arginase. Furthermore, the amide-N fraction of the liver protein of the carcass contained only slightly less N^{15} than the amidine fraction of the arginine, a finding which supports the idea that amide-N may possibly play a part as important as that of arginine in the synthetic mechanism (Leuthardt, 1938; Bach, 1939).

Bach (1939) suggested a new mechanism of urea synthesis in which glutamine was the principal ammonia carrier in place of ornithine and citrulline ('amide-N cycle') and explained the effect of citrulline on urea synthesis by its oxidation by keto-acids to glutamic acid and glutamine. He suggested a similar possibility for ornithine. Arginine and arginase were not considered to participate in this mechanism.

The abundant occurrence of arginase in mammalian liver, however, and the ease with which it can be demonstrated, may have influenced the general opinion for a long time in favour of arginine being the only source of urea. Clementi's empirical rule (Clementi, 1914) that arginase is present in the livers of ureatelic and absent from those of uricotelic animals, although it cannot be applied without limitations, has contributed much to this view. Leuthardt & Glasson (1942) attempted to reconcile the importance attributed by Leuthardt (1938) and Bach (1939) to glutamine with the essential role earlier given to arginine and arginase in urea synthesis. They suggested, though without much experimental evidence, an extension of the 'ornithine cycle' to include glutamine, which acts as ammonia carrier and transfers its ammonia to ornithine, whence the cycle continues to arginine and urea.

In view of these conflicting views on the problem of urea synthesis and the part played by arginine and arginase, an attempt was made to decide if urea synthesis could take place in the liver without the participation of arginase. Experiments to this effect were described by Bach & Williamson (1942) who showed that urea synthesis was not impeded when arginase was inhibited by excess ornithine. Details of this preliminary work are given in this paper, which also includes experiments on the nature of 'soluble' and 'tissue' arginase. Arginase is a highly soluble enzyme and will in experiments *in vitro* partially diffuse out of the tissue into the surrounding medium. Any effect observed with arginase might therefore be interpreted as originating from the extracellular part of the enzyme, the portion within the intact tissue not having come into play (Krebs, 1943). The participation of this 'tissue arginase' in the experiments, the similarity of 'tissue' and 'extracellular' arginase with respect to their Michaelis constant, to the effect of O_2 on the enzymes, and to the inhibition by ornithine, are shown in the first part of this paper, together with experiments on the accessibility of the tissue to arginine and ornithine. The remaining experiments are devoted to the main problem of the participation of arginase in urea synthesis.

METHODS

Animals. The rats used were similar to those previously employed (Bach, 1944).

Washing of slices. The method of slicing and weighing was also described in the above paper but the washing of the slices after weighing needs special attention. Each portion of slices was placed into a shallow 5 ml. dish, through which a steady stream of Ringer's solution was passed from a capillary tube. The dishes stood on a glass platform inside a crystallizing dish so that the overflow could be collected. After 5 min. washing in running Ringer's solution the dishes

were emptied, refilled with fresh Ringer's solution and the washing continued. This procedure was repeated and finally the slices were placed into fresh Ringer's solution before being transferred to the Barcroft vessels.

In this way it was possible to obtain from the same liver comparable portions of slices which were practically free from urea and preformed urea-forming substrates and which under the same experimental conditions yielded similar results. Many of the experiments described in this paper depend on the comparability of the different portions of slices used. Table 1 gives a few examples of duplicate experiments. All weights of tissue given in the text refer to wet weight.

Table 1. *Duplicate experiments with liver slices*

Wet wt. of slices (mg.)	Time of incubation (min.)	Arginine concentration (g./100 ml.)	Urea-N produced (μg.)
100	60	0·06	490 460
100	50	0·05	268 310
50	20	0·07	181 175
50	15	0·007	22 21
50	10	0·5	493 466
50	20	0·5	720 795
40	40	0·5	1060 1030

Incubation of slices. See Bach (1944). Tissue slices and tissue extracts were incubated at 38°.

Estimation of urea-N. Urea estimation was carried out as before (Bach, 1939) by incubation of the experimental fluid with urease followed by ammonia distillation at a low temperature *in vacuo* (Parnas & Heller, 1924). In the later experiments a Kjeldahl apparatus of the Pregl type (Markham, 1942) was used. When large amounts of urea-N were present the manometric method of Krebs & Henseleit (1932) was applied. With the Markham apparatus a distillation could be carried out within 2 min., a time sufficiently short to avoid decomposition of urea and amino-acids (see Table 2). With the Parnas apparatus the ammonia was distilled into 0·01 N-HCl, while with the Kjeldahl distillation into water was sufficient if the quantities distilled did not exceed 100 μg. The distillate was titrated with 0·01 N-alkali or 0·01 N-HCl respectively by means of a horizontal Conway microburette, as in the earlier paper (Bach, 1939).

Krebs's (1942) criticism of the method of microtitration, implying that a reading of 0·2 ml. on a microburette cannot be taken accurately enough to yield satisfactory results, is contrary to all experience of microtitration. Most experimenters agree that 0·005 ml., i.e. a 40 times smaller quantity, can be accurately read on a 1 ml. microburette. 0·2 ml. 0·01 N-HCl corresponds approx. to 100 μg. urea-N per sample (allowing for the fact that only 30–50 % of the whole is used for the titration); an error of $\pm$0·005 ml. will therefore correspond to $\pm$2·5 μg. urea-N. Conway (1939) calculated the standard deviation in titrating 1 ml. 0·01 N-acid with 0·01 N-alkali as 25×10^{-6} m.mol. = approx. 0·3 μg. N or (allowing for the fractional portion used) = 1 μg. per total sample. With quantities of 100 μg. these deviations amount to approx. $\pm$1–3 %. This error is roughly the same as in the recovery experiments (Table 2), in which known quantities of urea-N were estimated in absence and presence of tissue slices. Thus Krebs's explanation of discrepancies between his own results and those of Bach by attributing erroneous titrations to the latter is without foundation. Furthermore, to support his argument Krebs chose a few unusually divergent values from among fifty or more reasonably consistent controls. Incidentally, it is these particular results criticized which have been independently confirmed both by Gornal & Hunter (1943) and by Borsook & Dubnoff (1943).

Reagents. *l*(+)-Arginine was prepared from gelatin according to Whitmore (1932) and *l*(+)-ornithine from arginine according to Hunter (1939).

Preparation of liver extracts containing arginase

(*a*) *Glycerol extracts.* Fresh rat liver (4 g.) was ground up with glycerol (16 ml.). The mixture was centrifuged and filtered through glass-wool. In the anaerobic experiments of Figs. 2*b* and 3 the filtrate was transferred to a Thunberg tube which was then evacuated. Immediately before the incubation a measured fraction of its content was quickly transferred to Barcroft vessels containing the other reagents and irrigated with a N_2/CO_2 mixture.

(*b*) *Aqueous extracts.* Liver acetone powder (1 g.) was ground up with water (10 ml.). The mixture was centrifuged and filtered through glass-wool.

Table 2. *Ammonia and urea recovery experiments*

In presence of liver slices (mg.)	NH_3-N (μg.) Added	NH_3-N (μg.) Found	Urea-N (μg.) Added	Urea-N (μg.) Found	Error (%)	Apparatus used (P = Parnas K = Kjeldahl)
—	50	49·5	—	—	1	K
—	25·1	25·4	100	—	1	K
—	—	—	100	101·5	1·5	P
—	94·2	89·6	200	196	5 and 2	P
—	—	—	100	99·9	0	K
200	437·5	434	—	—	1	P
50	—	—	31	32·4	4·5	K
100	—	—	200	202	1	P
	In presence of 4 mg. ornithine, 6 mg. glutamic acid, and 6 mg. lactic acid:					
100	92	94·2	200	191	2 and 4·5	P
	In presence of 4 mg. ornithine and 2 mg. arginine:					
—	—	0	—	0	—	K

RESULTS

The participation of intracellular arginase in experiments with tissue slices

100 mg. liver slices were shaken in 5 ml. bicarbonate Ringer's solution in a Barcroft vessel, in absence of substrate, for 10 min. They were then transferred to another vessel containing fresh Ringer's solution and after 10 min. to a third and at 10 min. intervals to a fourth and fifth vessel. At the end of each 10 min. period arginine was added to the fluid from which the slices had been removed and the mixture was incubated for a further 10 min., after which the enzyme action was stopped by the addition of trichloroacetic acid and the urea formed was estimated. At the beginning of the fifth period of incubation arginine was added to the tissue suspension and the mixture was again incubated for 10 min. Any arginase found in solutions 1–4 must have diffused out of the tissue into the Ringer's solution during the first four incubations. Any arginase effect, however, found in the Ringer's solution of the fifth vessel must have been due both to intracellular and extracellular enzyme, since the substrate had been added in presence of the tissue.

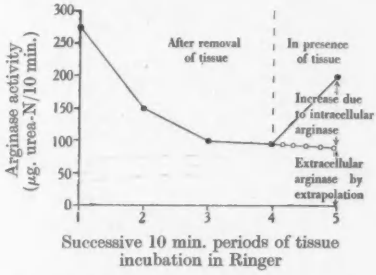
Arginase activity (μg. urea-N/10 min.)
After removal of tissue
In presence of tissue
Increase due to intracellular arginase
Extracellular arginase by extrapolation
Successive 10 min. periods of tissue incubation in Ringer

Fig. 1. The participation of intra- and extracellular arginase of liver slices. Arginine 0·5% (final concentration) added for activity determination: total volume 6 ml. One sample (100 mg.) liver slices incubated for 10 min. periods in Barcroft vessels each containing 5 ml. fresh Ringer. At the end of each of four periods 1 ml. arginine HCl (3%) added to the fluid after removal of the slices and further incubated = urea-N production from extracellular arginase. After the fifth period arginine HCl added without removal of the slices = urea production from extracellular plus intracellular arginase.

The amount of urea produced in each of the solutions is a measure of the outflow of arginase from the tissue during the corresponding period and is plotted in Fig. 1. It will be seen that the outflow gradually decreased during the first three incubations, when the level of the outflow of arginase became almost constant, and a similar urea-N value would have been expected after the fifth incubation had the arginine been added as in 1–4, namely after removal of the tissue. But it will be seen that, with tissue present, arginine hydrolysis in the fifth vessel rose to reach a new peak. This was obviously due to the participation of intracellular enzyme.

It can therefore be assumed that in such *in vitro* experiments 'tissue' arginase participates in the hydrolysis of arginine and also that the tissue is permeable to arginine.

Properties of arginase in liver slices and cell-free liver extracts

(*a*) *Michaelis constant* (K_m). Figs. 2*a* and 2*b* demonstrate the determination of K_m for arginase in liver slices and in cell-free glycerol extract of liver tissue.

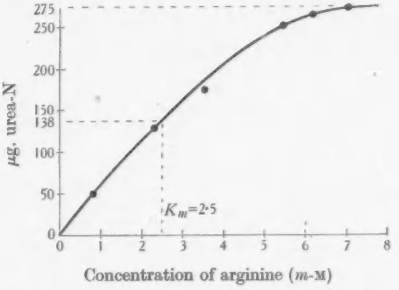

Fig. 2*a*. Determination of the Michaelis constant (K_m) for arginase in liver slices (50 mg.). Time of incubation 10 min. Total volume: 4 ml.

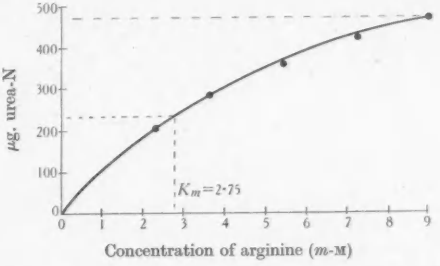

Fig. 2*b*. Determination of Michaelis constant (K_m) for arginase in glycerol extract (0·5 ml.) under anaerobic conditions. Time of incubation 10 min. Total volume: 4 ml.

The results, namely, K_m for 'tissue' arginase = $2{\cdot}5 \times 10^{-3}$ M and K_m for extract = $2{\cdot}75 \times 10^{-3}$ M-arginine, are as close as can be expected, considering the fact that each concentration of substrate had to be tested with a different portion of liver slices, and are consistent with the identity of the two forms.

(*b*) *The inhibitory effect of* O_2 *on arginase.* The effect of O_2 on arginase extracts, first observed by Edlbacher, Kraus & Leuthardt (1933), was studied with different O_2/N_2 mixtures in experiments with tissue slices and tissue extracts. In agreement with these workers, it was found that aqueous arginase extracts exposed to air were irreversibly damaged by O_2, while glycerol extracts were less affected. Further treatment with O_2 would therefore have little or no effect on an aqueous arginase extract previously exposed, while it should be possible to demonstrate O_2 inhibition with a glycerol extract prepared under anaerobic conditions. From Fig. 3

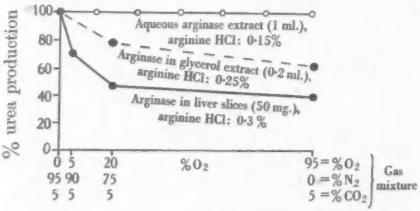

Fig. 3. Effect of oxygen on arginase. Time of incubation 30 min. Note: urea production under anaerobic conditions is taken as 100%. Total volume: 4 ml.

it can be seen that arginase in glycerol extract was increasingly inhibited by O_2 treatment, while no effect was seen with aqueous extracts. The effect of O_2 treatment on arginase in liver slices during incubation was even more marked, because the enzyme within the intact tissue was better protected against atmospheric O_2 by the cellular structure during the preparatory stages of the experiment, than was the enzyme extracted from the cells. So there is no doubt that both in tissues and in glycerol extracts arginase shows a higher activity under anaerobic conditions.

In experiments shown in Fig. 4 the irreversibility of O_2 inhibition was further illustrated. Two identical portions of slices, each divided into three equal parts, were incubated with arginine, one anaerobically and the other aerobically. For this purpose the Warburg vessels containing the tissue and the experimental mixture with arginine in the side-bulb were irrigated with the gas mixture for 10 min. both before and after being immersed in the water tank. 30–40 min. after arginine had been added to the experimental mixture, the first part of the aerobic and anaerobic portions were taken out and the urea produced by them was estimated. The second part of each portion was then incubated another 30–40 min. under the same conditions. The gaseous conditions of the third parts of each portion were reversed, i.e. the one which had been incubated under anaerobic conditions was irrigated with an O_2/CO_2 mixture for 10 min. and vice versa.

As a result it will be seen that in the initial period urea production was 2–3 times greater under anaerobic conditions than under aerobic; further that aeration of the anaerobic portion almost completely

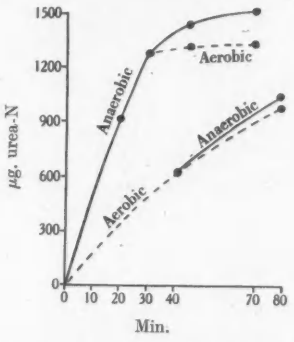

Fig. 4. Hydrolysis of arginine by arginase in liver slices (50 mg.) under anaerobic and aerobic conditions. Arginine HCl, 0·3%. Total volume: 5 ml.

inhibited any further urea production, while the replacement of O_2 in the aerobic portion by N_2 had scarcely any effect, the tissue being irreversibly damaged by shaking in 95% O_2.

Finally, in experiments shown in Fig. 5 the effect of O_2 on arginase is compared with that on the urea-synthesizing mechanism in liver slices. It will be

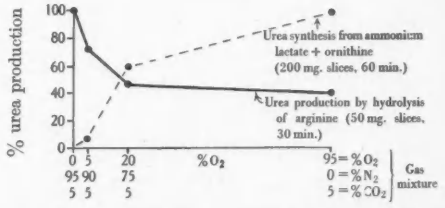

Fig. 5. The effect of O_2 on urea production by hydrolysis of arginine and by synthesis from ammonium lactate and ornithine in liver slices. Arginine HCl, 0·3%; ornithine HCl, 0·1%; lactate, 0·2%; NH_4Cl, 0·01%. Note: urea production by aerobic synthesis and by anaerobic hydrolysis is taken as 100%. Total volume: 4 ml.

seen that while arginase works most actively under anaerobic conditions and is gradually inhibited by O_2, urea synthesis is completely inhibited in absence of O_2 and increases with rising O_2 content of the gas mixture.

The conclusions to be drawn from the above experiments are twofold: (1) There is a close similarity between the behaviour of extracted and 'tissue' arginase as shown by their Michaelis constants and by the effect of O_2, so that cellular

environment apparently has little or no effect on these properties of the enzyme. (2) The enzyme within the intact cell structure is, however, better protected against atmospheric O_2 than the enzyme in aqueous solution.

(*c*) *Inhibition of arginase by ornithine.* In the search for a specific inhibitor of arginase the effect of ornithine was investigated. Its inhibitory action first observed by Gross (1920) on arginase in aqueous liver extracts can also be demonstrated on arginase in liver slices. The experiments shown in Fig. 6 give evidence for the similarity of the ornithine effect on tissue arginase and arginase extract of comparable strength.

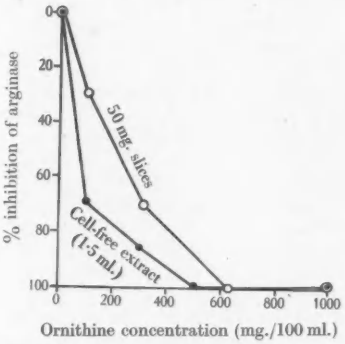

Fig. 6. The inhibition of arginase by various concentrations of ornithine in liver slices and cell-free extract. Arginine HCl, 75 mg./100 ml. Time of incubation 20 min. Total volume: 4 ml.

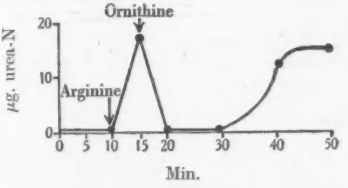

Fig. 7. The inhibition of arginase by excess ornithine in liver slices (progress curve). 50 mg. slices; arginine HCl, 15 mg./100 ml.; ornithine, 1·6%. Total volume: 5 ml.

The lower concentration of ornithine needed to inhibit arginase in extracts than in tissue slices raises the problem whether intracellular arginase is accessible to ornithine added to the suspension of tissue slices. Experiments shown in Fig. 7 made it clear that there was an immediate response after either arginine or ornithine had been added to the experimental mixture. Since the inhibition of arginase by ornithine is complete, and since the above experiments have shown that intracellular arginase participates in these *in vitro* reactions, both substrates must have had immediate access to the tissue cells and the fact that no time lag was observed is evidence against a low tissue permeability for either.

(*d*) *The fate of ornithine in liver metabolism.* It will be seen, however, that the inhibitory effect of ornithine is greatly reduced after prolonged incubation with tissue slices (Figs. 7, 9), while it remains almost constant in arginase extracts as shown in the experiments of Fig. 8. Table 3, section A, further

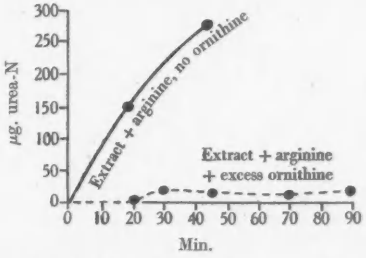

Fig. 8. The inhibition of arginase by excess ornithine in cell-free aqueous extract (progress curve). Arginine HCl, 75 mg./100 ml.; ornithine HCl, 0·48%; aqueous extract, 0·5 ml. Total volume: 5 ml.

illustrates this phenomenon. The results of section B of Table 3 are particularly interesting: when ornithine is pre-incubated with the tissue before arginine

Table 3. *Inhibition of arginase by excess ornithine*

(A) Effect of the period of incubation

Exp. no.	Arginase in	Ornithine added (g./100 ml.)	Period of incubation (min.)	Inhibition (%)
1	Liver slices	1·2	45	77
2	Liver slices	1·2	120	28
3	Cell-free extract	0·14	20	64
4	Cell-free extract	0·14	90	63

(B) Effect of time and temperature of pre-incubation of ornithine with liver slices

Exp. no.	Ornithine (g./100 ml.)	Pre-incubation min.	Pre-incubation temp.	Inhibition (%)
5	1	Not pre-incubated		76
6	1	30	18°	57
7	0·7	30	18°	51
8	0·7	30	37°	24

Note. In Exps. 1–5 ornithine and arginine were added simultaneously. In Exps. 6–8 arginine was added after pre-incubation of ornithine with the tissue.

is added, its inhibitory effect on arginase in the following period of incubation is reduced and this reduction is enhanced if pre-incubation is carried out at a higher temperature. From these results it is possible to conclude that ornithine is being

metabolized by enzymic systems present in the intact liver tissue but absent in liver extracts. If, therefore, ornithine were exposed to such a metabolic attack before or after arginase comes into play it would be partly used up and, with the decreasing concentration of the inhibitor, arginase becomes less and less impeded. A higher temperature would intensify any such metabolic action.

Urea synthesis with inhibited arginase

The strong inhibitory action of excess ornithine on the arginase of liver slices can be used to throw light on the problem of the participation of arginase in the synthesis of urea in the liver. For if arginase were considered indispensable for the synthesis of urea an inhibition of the enzyme should also stop the formation of urea. In the experiments recorded in Fig. 9 four portions of liver slices, each weighing 200 mg., were incubated in bicarbonate Ringer's solution (curves 1–4).

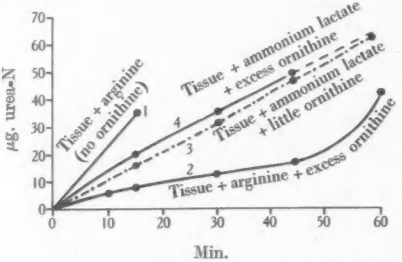

Fig. 9. Urea synthesis with and without inhibition of arginase. 200 mg. liver slices; arginine, 7·5 mg./100 ml.; ornithine, 0·4 and 1·6% respectively; Na lactate, 0·2%; NH_4Cl, 0·01%. Total volume: 5 ml.

For curve 1 arginine was added to demonstrate the presence of arginase; for curve 2 arginine and excess ornithine were added, as a result of which urea production was strongly inhibited in the initial period. In the experiments for curve 3 the Ringer contained ammonium lactate and small quantities of ornithine in concentrations identical with those used by Krebs & Henseleit (1932) for urea synthesis in liver slices. It will be seen that 60 μg. urea-N were formed in 60 min. In the experiments for curve 4 the composition of the experimental mixture was identical with that of the experiments for curve 3, except that excess ornithine in quantities equal to those used for the inhibition of arginase were added.

Curve 4 shows that in spite of strong inhibition of arginase in the first 30–40 min. (see curve 2) the synthesis of urea was entirely unimpeded or even slightly accelerated in comparison with that shown in curve 3, where arginase was not inhibited.

Arginine content of liver slices

Urea production from arginine with both uninhibited and inhibited arginase as shown in curves 1 and 2 of Fig. 9 depends, amongst other things, on the quantities of arginine added. With too small a quantity of arginine used in the experiments for curve 2 inhibition would appear to be unduly enhanced and may not have corresponded to physiological conditions. It was therefore decided to choose a concentration of arginine not below but rather above physiological level. The actual concentration of arginine in the tissue during urea synthesis was therefore estimated.

500 mg. thoroughly washed slices were incubated for 30 min. with ammonium-lactate and ornithine in optimal concentration (Krebs & Henseleit, 1932) for urea synthesis. At the end of this period of incubation the urea formed was estimated and a measured portion of the experimental fluid, free from slices, was transferred to another vessel for a second period of incubation (45 min.) with a cell-free liver extract containing arginase. The activity of this enzyme preparation had previously been tested and its urea content determined. The experimental mixture used in the second incubation thus contained initially a known fraction of (*a*) urea synthesized during the first period of incubation, plus (*b*) urea initially present in the tissue extract. Any quantity in excess of (*a*) + (*b*) found at the end of the second period of incubation must have been formed in this period by the action of arginase on any arginine present. Since the arginase extract was arginine-free any arginine found must have originated from the first experimental fluid used for the incubation of the tissue slices.

Table 4 gives examples of the experiments described above: the example showing the higher arginine content corresponds with the presence of 220 μg. arginine HCl/500 mg. tissue or 88 μg./200 mg.

Table 4. *Determination of the arginine content of liver slices* (500 mg.) *during urea synthesis*

	μg. urea-N found				
Initial incubation period (min.)	(1) After initial period of incubation	(2) After second period of incubation	(3) In arginase extract used for second incubation	Urea-N formed by hydrolysis of tissue arginine: (2) minus (1 + 3)	Urea-N expressed as arginine HCl (μg.)
30	65	111·5	17	29·5	220
90	168	191	17	6·0	45

tissue. In the experiments shown in Fig. 9, 375 μg. arginine HCl were added to 200 mg. tissue (curves 1–2), i.e. more than four times the quantity found according to Table 4.

The main conclusion drawn from the experiments in Fig. 9 must be that arginase does not necessarily participate in the urea synthesis. This conclusion is based on the fact that, with the arginase activity inhibited, urea production from arginine occurs at a low rate, whilst on the other hand that from ammonium lactate is unimpeded. This discrepancy would be even more marked if the arginine concentration for curve 2 were to be still further reduced.

DISCUSSION

Participation of 'tissue' arginase in the experiments. The above experiments were designed to prove that urea synthesis from ammonium lactate remains unimpeded when the arginase in the tissue is strongly inhibited by excess ornithine. However, evidence had to be given that ornithine inhibited not only the arginase which diffused from the cells into the surrounding medium, but also the enzyme within the intact tissue. For this purpose it was first shown that the 'tissue arginase' participates in the hydrolysis of arginine in liver slice experiments (Fig. 1) and hence that the tissue is permeable to arginine, and further that the activity of arginase in liver slice experiments, like that in tissue extracts, can be fully inhibited by ornithine. This proves that the tissue is also permeable to ornithine. The evidence is supplemented by experiments showing that the enzyme had similar properties whether outside or inside the intact cellular structure. Thus a similar Michaelis constant and a similar behaviour towards oxygen and ornithine was found for 'tissue' arginase and cell-free arginase.

Inhibitory effect of ornithine on arginase. The effect of ornithine on arginase is specific and is caused by mass action in shifting towards the left the equilibrium in the reaction: arginine $\rightleftharpoons$ ornithine + urea. Urea has a similar inhibitory effect on arginase (Vorochenko, 1936).

Metabolism of ornithine. Independently of this inhibitory action on arginase, ornithine is probably metabolized by the intact tissue but not by aqueous tissue extracts. For this assumption only indirect evidence could be secured by the findings that the inhibiting power of ornithine decreases with longer periods and higher temperature of incubation. The fate of ornithine in liver metabolism is a problem apart from its possible connexion with urea synthesis. Work in this direction is in progress.

Effect of O_2 on arginase. The irreversible inhibitory effect of O_2 on arginase in tissue extracts previously described by Edlbacher *et al.* (1933) was also observed with arginase in liver slices. Whereas tissue arginase appears to be extremely sensitive towards O_2—the activity of arginase in yeast can even be fully suppressed by aeration (Edlbacher, Becker & v. Segesser, 1938)—urea synthesis greatly depends on an ample supply of O_2. This is clearly illustrated in experiments shown in Fig. 5 from which it can be seen that the oxygenation conditions under which urea synthesis takes place are unfavourable for arginase, while urea synthesis is entirely suppressed at low oxygen pressures almost optimal for the activity of arginase. While it is improbable that there is no intermediate condition under which both reactions may take place simultaneously, the experiments reported are not very favourable for the postulate that arginase is an obligatory intermediate enzyme in the synthesis of urea.

Participation of arginase in urea synthesis. Fig. 9 gives the crucial experiments bearing on the question of the participation of arginase in the synthesis. Even with an arginine concentration four times as strong as can be expected to exist in the tissue (Table 4), urea production from arginine with inhibited arginase (curve 3) cannot account to any significant extent for the synthesis of urea from ammonium lactate with arginase inhibited to the same degree (curve 2). These experiments were repeated with the same result: in both cases urea synthesis with inhibited arginase was slightly faster than with uninhibited arginase. The results clearly indicate the existence of a synthetic mechanism for urea other than, or in addition to, that of the 'ornithine cycle'.*

Current ideas on the mechanism for the synthesis of urea. The results described in this paper may encourage the search for a new mechanism for the synthesis of urea. Whether there is any truth in the synthetic schemes suggested hitherto by Krebs & Henseleit (1932) or by Bach (1939) or others can only be finally decided by the chemical isolation of the reaction products. So far this has not been achieved for any scheme and indirect methods are

* Krebs (1943) postulated that under the conditions of Bach & Williamson (1942) only the small, extracellular part of the 'potential' arginase of the tissue participates in arginine hydrolysis and can be inhibited by ornithine. However, the participation of 'tissue arginase' has been demonstrated here.

He further argues that the remainder was uninhibited and participated in urea synthesis. Apart from the fact that it is difficult to know what importance can be attached to a calculation of the 'potential' activity of an enzyme (part of which had been removed by extensive washing) within the cellular structure from measurements on unwashed disintegrated cells, any such deduction cannot invalidate these experiments. For there is no ground for assuming that under the same experimental conditions the arginase hydrolyzing arginine supplied from outside the cell is different in nature from that hydrolyzing arginine synthesized within it from added ornithine. Therefore, if the observable proportion of extracellular plus intracellular arginase, however great or small, is inhibited by ornithine, it must be assumed that the arginase available for the synthesis observed under exactly the same experimental conditions is inhibited to the same degree.

rarely safe enough to decide the issue. The results of Gornal & Hunter (1943), who found accumulation of citrulline with added ornithine in tissue slices, suffer from the unspecificity of the colorimetric estimations of citrulline (the Fearon (1939) reaction works with all mono-substituted ureas) and from the high control values amounting in some cases to 60–80 % of the total changes (see Gornal & Hunter, 1943, Table IV). However, these workers and also Borsook & Dubnoff (1943) confirm Bach's findings (1939) that the ratio of urea-N found to ammonia-N disappeared was 1, instead of 2 as expected from Krebs's theory.

The present stage of knowledge with regard to the 'ornithine cycle' is well summarized by Borsook & Dubnoff (1943). Although, according to the authors, 'there is increasing expression of doubt regarding the ornithine cycle', there is in our mind always the possibility that the mechanism involving arginase exists side by side with others which may be called into action according to the conditions prevailing in the synthesizing tissue at the time.

Alternative role of arginase. Finally, a role of arginase, alternative to that as the principal catalyst in the synthesis of urea, may be considered. Cases are known where no parallelism is observed between the arginase content of a tissue and its urea production. Takahara (1938), for example, found a marked decrease in the arginase content of liver and a simultaneous rise of the blood-urea level after phosphorus poisoning, and Edlbacher & Koller (1934) report very high arginase contents of liver sarcoma coupled with incapacity to synthesize urea. According to Edlbacher & Merz (1927), arginase activity in malignant tumours does not represent a partial reaction in the synthesis of urea but may be considered as a specific growth factor, or may even participate in protein synthesis by the transfer of N to non-nitrogenous substances (Edlbacher & Baur, 1938).

SUMMARY

1. A specific inhibition of arginase in tissue slices was obtained by adding large quantities of ornithine.

2. Urea synthesis in liver slices from ammonium lactate takes place unimpeded when arginase is thus inhibited.

3. While the arginase activity in liver slices is irreversibly inhibited by O_2 and is optimal under anaerobic conditions, urea synthesis from ammonium lactate depends on ample oxygenation.

4. These findings support the idea of a synthesizing mechanism other than, but not necessarily excluding, the 'ornithine cycle'. This new mechanism is independent of arginase.

5. Evidence was obtained that the tissue arginase, as well as that which passed into the solution, participated in the experiments described.

6. The different theories of the mechanism of urea synthesis are discussed and an alternative role of arginase is indicated.

The experiments shown in Figs. 3, 4 and 5 were carried out in collaboration with Dr H. Laser, Molteno Institute, Cambridge, to whom we wish to express our thanks for his valuable help.

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The Oxidative Metabolism of *Ascaris suis*

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Bunge (1883–4) stated that *Ascaris mystax* is able to live for 5–6 days under anaerobic conditions in 1% saline solution. At that time the intestinal gases were considered to be entirely free of oxygen. Bunge, although inclined to attribute to *Ascaris* a strictly anaerobic mode of life, observed that worms kept in air lived, as a rule, longer than those under anaerobic conditions. He therefore concluded that *Ascaris* was probably unable to live in complete absence of O_2, but that the O_2 requirement was very small. Bunge later (1890) confirmed these results with *A. acus* and *A. lumbricoides*. His results, however, have constantly been misquoted (Weinland, 1901; Schulte, 1917; Krummacher, 1919 and Jordan, 1929) as having proved the obligatory anaerobiosis of *Ascaris*. Weinland goes so far as to dismiss some of his own results which are not in accordance with strict anaerobiosis; having found that the CO_2 production of worms was much greater under aerobic than under anaerobic conditions, he rejected the possibility that this increase might have been due to respiratory CO_2 but attributed it to the formation of eggs or bacterial action. Jordan even quotes the authority of Bunge for stating that 'O_2 is a poison for *Ascaris* and cannot be utilized by them for oxidative processes'. Slater (1925), however, drew attention to the facts that the physiology of digestion and the anatomical picture inside the gut, especially in proximity to the portal vein, do not justify the assumption of complete absence of O_2 in the intestine. Long & Fenger (1917) had already demonstrated that the intestinal gases contain on an average about 5% O_2 (by vol.). Slater (1925) speaks of a 'suppressed animation' of *Ascaris* in absence of O_2, the avoidance of movements enabling the worms to live for some time in inert gases. He showed that *Ascaris* exposed to repeated electric stimuli soon lose their ability to react when they are maintained in an atmosphere of H_2 but not in air. He is of the opinion that O_2 is necessary for the normal life of *Ascaris*.

Later workers (Adam, 1932; Harnisch, 1932 *a*, *b*, 1933; v. Brand, 1934–5; Friedheim & Baer, 1933; Krüger, 1935) have determined by direct measurement and with different methods the O_2 consumption of *Ascaris*. Although individual differences are considerable, all the determinations agree on a mean O_2 uptake of about 80 μl./g. wet wt./hr. at 39° in air (range 65–100 μl.). Harnisch (1933), who determined the O_2 consumption of 1 cm. lengths of the anterior end of worms at O_2 tensions varying from 0·8 to 95% O_2, found that the magnitude of O_2 uptake depends on the O_2 tension, rising considerably with increasing O_2 tension and being about four times as great in 95% O_2 as in air.

Harnisch also observed that the CO_2 output at different O_2 tensions remains constant although the magnitude of O_2 uptake varies considerably. He maintained that no respiratory CO_2 is formed (respiratory quotient (R.Q.) = 0), but v. Brand found the R.Q. = 0·9.

In this paper an attempt is made to analyze the ability of *A. suis* to utilize O_2.

METHODS

The O_2 uptake and, in some cases, the CO_2 output of whole worms or muscle pulp were measured in open-type Warburg manometers at 39°. The media used were either 0·05 M-phosphate buffer, pH 7·3, or Ringer phosphate solution. No difference was found attributable to these different media. For work with whole worms small specimens, usually males, were chosen. For worms of 1–2 g. wet wt. flasks of 18–20 ml. capacity and for larger worms (3–4 g.) flasks of about 28 ml. were used. For the calculation of the vessel constants the weight of the worms was considered as extra liquid, and added to the volume of the fluid, since determination of the density of the worms showed that this was virtually 1.

When substrates were added to the medium the worms were first injected with an amount of substrate sufficient to establish within them the same substrate concentration as in the outside medium. In this way no complication due to the rate of diffusion of substrate into the worm arose. A small amount of body fluid was withdrawn from the worm by means of a syringe and either reinjected into the worm after the substrate had been dissolved in it, or the body fluid of another worm, suitably adjusted with substrate, was injected.

Muscle pulp was prepared in the following way. Larger worms, of about 6 g., were cut open longitudinally and freed from intestine, eggs, etc. The muscular layer was then scraped from the cuticle and ground with a little sand in 6 ml. medium per worm. The sand was removed by slight centrifugation. The supernatant pinkish, opaque fluid contained the finely dispersed muscle pulp. 0·5–1·0 ml. of this was used per vessel and made up to 3·0 ml. with buffer, substrate and other reagents. The muscle pulp keeps fairly well in the ice-chest for several days with only slight loss of its oxidative capacity.

Coenzyme-free muscle pulp. 10 ml. of muscle pulp prepared as before were diluted with buffer or water to 100 ml. and brought to pH 5 with acetic acid. A slight precipitate

which formed within ½ hr. at room temperature was centrifuged, resuspended in 100 ml. medium and precipitated a second time at pH 5. This was again centrifuged and was suspended in 10 ml. of neutral buffer solution. Its aerobic oxidative power was lost within 2–3 days, even when kept in the cold, but its power to reduce methylene blue (M.B.) anaerobically in the presence of sodium succinate remained unimpaired much longer.

RESULTS

O_2 uptake of whole worms and of muscle pulp. In agreement with the earlier results recorded in the introduction, the O_2 uptake of whole worms in air was found to be about 80 µl./g. wet wt./hr. at 39°. The O_2 uptake of the pulp, calculated/1 mg. dry wt. of the pulp/hr. (Q_{O_2}), was about 1·3.

Addition of substrates. Substrates, such as glucose (0·2 %), sodium lactate (0·02 M) and sodium succinate (0·02 M), added to the muscle pulp or injected into worms, failed to increase their O_2 uptake.

Influence of O_2 tension. The magnitude of the O_2 uptake of whole worms is dependent on the O_2 tension. In 5 % O_2 the O_2 uptake is half that in air, and in 100 % O_2 it is about three times higher than that in air (Fig. 1). High O_2 tension, however, is

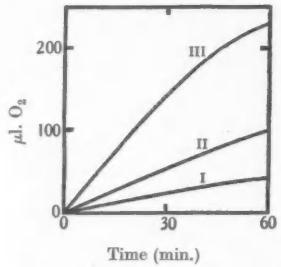

Fig. 1. O_2 uptake of whole worm (µl./g.) at different O_2 tensions. I, 5 % O_2, 95 % N_2; II, air; III, 100 % O_2.

toxic to the worms, causing their death within about 1 hr. The reasons for this effect will be discussed later.

O_2 debt of whole worms. Worms brought after 17–20 hr. anaerobiosis into 5 % O_2 in N_2 or into air showed an increase of the O_2 uptake by 60–100 % when compared with worms of the same batch which had previously been kept in air (Fig. 2). This increase in O_2 uptake develops during the first ½ hr., remains for about 2 hr. and then gradually decreases to the values of the controls.

Addition of M.B. and the succinic system. 0·5 ml. of muscle pulp made up with buffer to 3·0 ml. reduces 0·3 ml. M.B. (0·4 mg./ml.) anaerobically within 3–4 min. The addition of succinate shortens the reduction time to 1–1½ min. The addition of M.B. to muscle pulp in the absence of added substrate raises the O_2 uptake: thus M.B. is here acting as an O_2 carrier in the oxidation of some undetermined substrate present in the muscle pulp.

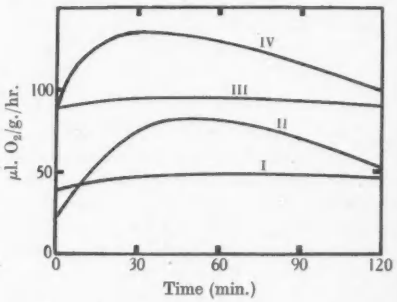

Fig. 2. Effect of anaerobiosis on subsequent O_2 uptake of whole worms (µl./g./hr.) at different O_2 tensions. I and II, 5 % O_2, 95 % N_2; III and IV, air; II and IV, following 18 hr. anaerobiosis; I and III had been kept in air.

While the addition of succinate to muscle pulp does not raise the O_2 uptake, the simultaneous presence of succinate and M.B. produces a considerable increase. This shows that part of the succinic system is present in muscle pulp but that an available O_2 carrier is lacking. The addition of cytochrome *c* or of riboflavin to muscle pulp in the presence of succinate does not, however, increase the O_2 uptake (Fig. 3).

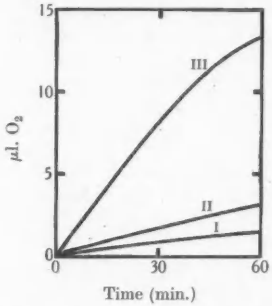

Fig. 3. O_2 uptake (µl./mg. dry wt.) of muscle pulp in air. Additions: I, none and sodium succinate only; II, M.B. only; III, sodium succinate and M.B. together.

Coenzyme-free muscle pulp. Oxaloacetic acid is a strong inhibitor of succinic dehydrogenase (Das, 1937). It is a normal intermediate in the oxidation of succinic acid by higher animals. Keilin & Hartree (1940) have shown that the inhibition of the cytochrome-succinic system by coenzyme I is due to the formation of oxaloacetic acid from fumaric via malic acids. A coenzyme-free muscle pulp was therefore prepared, which had lost the ability to form oxaloacetic acid. In the presence of M.B. and succinate,

the O_2 uptake (Q_{O_2}) of this preparation was twice as high as that of untreated muscle pulp (Fig. 4).

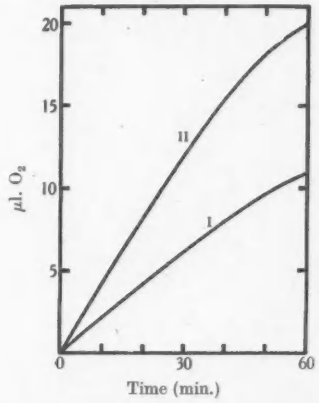

Fig. 4. O_2 uptake (μl./mg. dry wt.) of muscle pulp (I) and of coenzyme-free muscle pulp (II) in presence of sodium succinate and M.B. in air.

Influence of catalase. The addition of catalase to muscle pulp considerably raised the O_2 uptake. A still further increase was obtained when catalase and M.B. were added together (Fig. 5). *Ascaris* is

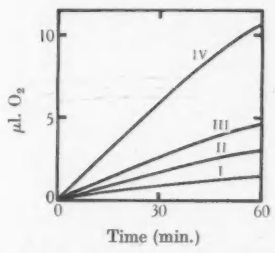

Fig. 5. O_2 uptake of muscle pulp (μl./mg. dry wt.) in air. Additions: I, none; II, M.B.; III, catalase; IV, M.B. and catalase.

known to contain very little catalase. The catalase content of the body of *Ascaris* was found to be only 1/500th of that of the same weight of mammalian blood corpuscles. The fact that the addition of catalase to muscle pulp increased the O_2 uptake suggested that H_2O_2 may have been formed during the oxidation of some substrate, and in the absence of catalase may have destroyed the enzyme concerned.

The formation of H_2O_2 in muscle pulp. The formation of H_2O_2 in a primary oxidation reaction can be detected easily by its utilization in a coupled oxidation of ethanol to aldehyde catalyzed by catalase (Keilin & Hartree, 1935–6). Fig. 6 shows that the addition of either ethanol or catalase alone to muscle pulp in air and in the presence of M.B. and sodium succinate has practically no effect on the rate of O_2 uptake, while the addition of both catalase and ethanol doubles the O_2 uptake of the primary oxida-

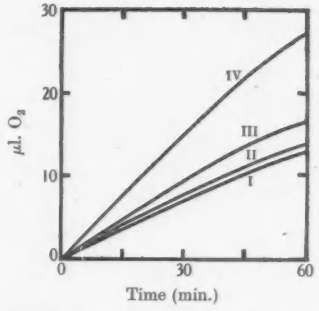

Fig. 6. O_2 uptake of muscle pulp (μl./mg. dry wt.) in air. Sodium succinate and M.B. were present in all samples. Further additions: I, none; II, ethanol; III, catalase; IV, ethanol and catalase.

tion reaction. Similar results with regard to coupled oxidation were obtained when coenzyme-free muscle pulp was used instead of untreated muscle pulp. Catalase, however, produced here a greater increase in O_2 uptake than with ordinary pulp, owing to the fact that coenzyme-free muscle pulp is practically free of catalase (Fig. 7).

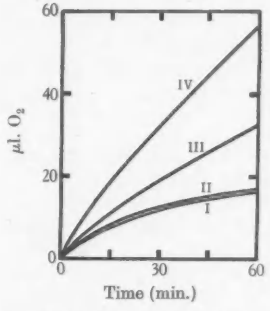

Fig. 7. O_2 uptake of coenzyme-free muscle pulp (μl./mg. dry wt.) in air. Sodium succinate and M.B. were present in all samples. Further additions: I, none; II, ethanol; III, catalase; IV, ethanol and catalase.

The addition of the unphysiological O_2 carrier, such as M.B., might be held responsible for the formation of H_2O_2; hence it was determined if coupled oxidation occurs also in the absence of M.B. No effect was obtained when catalase and ethanol were added to muscle pulp at the O_2 tension of air.

In 100 % O_2, however, which raised the O_2 uptake of the controls by 80 % as compared with that in air, coupled oxidation took place, as was shown not only by the increased O_2 uptake (Fig. 8) but also by a brownish coloration of the KOH-soaked filter

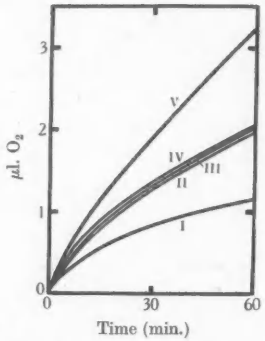

Fig. 8. O_2 uptake of muscle pulp (μl./mg. dry wt.) in air and in 100% O_2. I, air, no addition; II–V, 100% O_2. Additions: II, none; III, ethanol; IV, catalase; V, ethanol and catalase.

paper used for absorption of CO_2. This discoloration is due to polymerization of aldehyde, and proves that ethanol has been oxidized by peroxide in a secondary oxidation.

Formation of H_2O_2 *in the whole worm.* The fact that the raising of the oxidation rate of muscle pulp by the addition of M.B., or by an increase of the O_2 tension, caused the formation of H_2O_2, seemed to offer an explanation for the observation that worms die fairly quickly when brought into an atmosphere of pure O_2. Death appears with characteristic symptoms, the worms becoming very active, often bending their bodies in vertical loops. After about 30–60 min. they seem to be in a state of rigid spasm, with their loops, especially at the posterior end, well maintained. They then die in this spasm, which persists for some time after death. In addition, they quickly change colour from pink to yellowish white.

Experiments were undertaken to see whether the formation of H_2O_2 could be proved in such worms. This was done in two ways: (1) by peroxidatic oxidation of *p*-phenylenediamine, (2) by the oxidation of oxyhaemoglobin to methaemoglobin.

Worms were suspended in Erlenmeyer flasks in 20 ml. buffer per worm, to which a small amount of *p*-phenylenediamine and 1–2 drops of a purified peroxidase preparation were added. The flasks were then filled with O_2 and incubated at 39°. After ½–1 hr. a blue colour began gradually to develop in the medium, turning to dark blue within 2–3 hr. This indicates the presence of H_2O_2 and its gradual and increasing appearance in the medium by diffusion out of the dying or dead worm. The blue colour is quite different from the spontaneous darkening of the medium to a burgundy shade which appears in the control flasks, containing the same medium but no worms. The blue colour did not develop when worms were kept in the same medium under anaerobic conditions.

That the colour change from pink to whitish yellow of the worms when exposed to O_2 is due to the oxidation of haemoglobin to methaemoglobin by H_2O_2 was demonstrated in the following way. Worms were injected with chicken oxyhaemoglobin, which contains only a little catalase, and were exposed to O_2 hypertension. The colour of the worms changed rapidly from red to brown because of the complete oxidation of the injected oxyhaemoglobin to methaemoglobin.

Respiratory quotient (R.Q.) *of whole worms in air and* 100 % O_2. From a large batch of worms two pairs of equal weight were chosen. Two Warburg vessels containing one worm each of a pair were filled with air, and two vessels, containing the other two, with O_2. One vessel of each pair contained KOH for the measurement of the O_2 uptake, while in the others KOH was omitted. On the assumption that the O_2 uptake was equal in both vessels of each pair, the R.Q. can be calculated. In experiments of 1 hr. duration the R.Q. was found to be 1·1–1·2 in air and 0·5–0·6 in O_2.

The O_2 uptake in O_2, as already stated, is at the beginning of the experiment about 300 % of that in air and it falls within 2 hr. to zero or to a very low level. It continues, however, in all cases, and is sometimes quite large, after the apparent death of the worm. This is most probably due to post-mortem autolytic oxidative reactions and not to respiration. The worm's death which, as stated in the literature, is sometimes difficult to determine, was assumed to have occurred when rapid warming of the worm from room temperature to 39° or 42° failed to induce any movements.

Influence of KCN *and* NaN_3. Neither KCN (0·002–0·01 M) nor sodium azide (0·001–0·004M) affects the O_2 uptake of muscle pulp with or without addition of substrate, nor do they prevent the rise of O_2 uptake on addition of M.B. (Fig. 9). On the contrary, high concentrations of KCN (0·01 M) in the presence of M.B. increase the O_2 uptake still further than M.B. alone. This must be considered due to the fact that KCN, as a ketone fixative, combines with oxaloacetic acid, rendering it incapable of inhibiting succinic dehydrogenase (Green & Dewan, 1937). This case is comparable to the increased O_2 uptake by coenzyme-free muscle pulp, described above.

The respiration of whole worms is also unaffected by KCN in as high a concentration as 0·01 M and,

to a lesser degree, by NaN_3 (0·004 M). In air the worms show, in the presence of KCN, an O_2 uptake which remains normal for a long time and they, as a rule, remain alive for at least 24 hr. With NaN_3

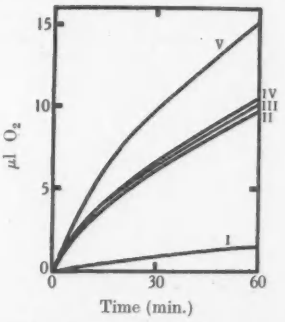

Fig. 9. O_2 uptake of muscle pulp (μl./mg. dry wt.) in air. Sodium succinate was present in all samples. Further additions: II, M.B.; III, M.B. and NaN_3 (0·004 M); IV, M.B. and KCN (0·002 M); V, M.B. and KCN (0·01 M).

worms die within about 3 hr. For the first 60–90 min. their O_2 uptake is very little affected, but later drops rapidly to zero (Fig. 10). The fact that even after injection into the worm of NaN_3 (final concentration in the worm 0·004 M) there is no immediate inhibition

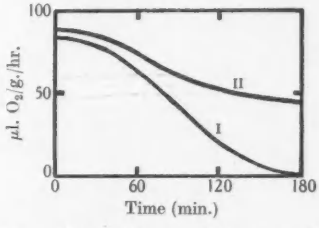

Fig. 10. O_2 uptake of whole worm (μl./g. wet wt./hr.) in air. I, NaN_3 (0·004 M); II, KCN (0·01 M).

of respiration seems to suggest that death is not due to the poisoning of the respiratory system but to other secondary effects, e.g. paralysis of the nervous system. The same applies to a lesser extent to the influence of KCN.

DISCUSSION

The O_2 uptake in air of the whole worm is, on an average, 80 μl./g. wet wt./hr. and the Q_{O_2} of the worm muscle pulp is 1·3. The addition of substrates, such as glucose, lactate or succinate does not increase these figures. Although, in view of the great activity of the worms at 39°, this appears to be a small rate of O_2 uptake, it is nevertheless of the same magnitude as that of the human body. The O_2 uptake of man at rest is of the order of 200 μl./g./hr., while that of the worms in air is as much as $\frac{2}{5}$–$\frac{1}{2}$ of this rate. The low Q_{O_2} (1·3) of muscle pulp hardly differs from that of mammalian muscle pulp. On the other hand, the rate of O_2 uptake of the worms is strictly dependent on the O_2 tension, which is not the case with mammalian tissues. Normally at the low O_2 tension in the intestine of the host, the O_2 uptake of the worm will be much lower than 80 μl./g./hr., and this seems to justify Bunge's conclusion that the O_2 requirement of *Ascaris* is very small. Is the worm a facultative aerobic organism, or is the O_2 uptake, however small, necessary? The second viewpoint, i.e. the necessity of aerobiosis, is supported by the evidence that the O_2 uptake is quite considerable even at the low O_2 tension of 5% by vol. and also by the appearance of an O_2 debt after prolonged anaerobiosis. Although the magnitude of the O_2 debt does not seem to be impressive, it must be considered in relation to the very small O_2 requirement.

The low O_2 requirement seems to be conditioned by, or to have conditioned, a system which is particularly adapted to allow only low rates of oxidation. Very little cytochrome is present. The fact that the addition of cytochrome *c* or of riboflavin to pulp did not increase the O_2 uptake may be explained by the large molecular size of cytochrome which does not permit its entry into the cells and by the assumption that riboflavin also does not readily enter. Methylene blue, which is readily diffusible, acts as an effective O_2 carrier and raises the O_2 consumption. Another brake on the O_2 consumption is probably due to the apparent inability of *Ascaris* to decompose oxaloacetic acid which, when formed, poisons succinic dehydrogenase and in this way indirectly depresses the oxidation of succinic acid. This was proved by two facts: (1) a coenzyme-free muscle pulp, which is unable to form oxaloacetic acid, shows a much increased O_2 uptake, and (2) if oxaloacetic acid is removed by combination with KCN the O_2 uptake rises considerably.

Every increase of the O_2 uptake, whether brought about by M.B. in air or by an increase of the O_2 tension, causes the formation of H_2O_2, which is a powerful cell poison. Its presence was proved in pulp by coupled oxidation on addition of catalase and ethanol, and in whole worms by the oxidation of *p*-phenylenediamine in the presence of peroxidase and the oxidation of injected oxyhaemoglobin to methaemoglobin. Owing to the fact that the worms contain very little catalase, they are unable to decompose H_2O_2 sufficiently rapidly to render it harmless. Whether at their normal low oxidation rate no H_2O_2 at all is formed, or only so little as can be dealt with by the worms, cannot be decided. It seems unlikely, however, that by simply increasing the O_2 tension a type of respiration whereby O_2 is re-

duced to H_2O should be changed to another type whereby O_2 is reduced to H_2O_2. The fact, however, that methaemoglobin, which is a weak peroxidase, is formed under the influence of H_2O_2, makes it feasible that some H_2O_2, if produced at low oxidation rates, may be eliminated by secondary peroxidatic oxidations catalyzed by methaemoglobin.

The death and change of colour of worms exposed to pure O_2 are assumed to be due to the formation of H_2O_2, the colour change being due to the formation of methaemoglobin.

The determinations of the R.Q. in air and in O_2 are open to criticism. The rate of respiration of two worms of equal weight is not necessarily the same, and still less so as one worm respires in almost complete absence of CO_2, while the other worm is in an atmosphere of increasing CO_2 tension. Differences in the oxidative rate with differing CO_2 tensions exist with other tissues (Laser, 1942; Craig & Beecher, 1943). It is furthermore possible that some of the CO_2 evolved is not respiratory CO_2 but is expelled by fixed acids from the bicarbonate present in the worm. The bicarbonate content of the worm, as used under the conditions of these experiments, was found to be very low, 30 μl./ml. body fluid, or per g. wet wt. As the worm produces constantly comparatively large amounts of fixed acid, it must be assumed to possess a fairly efficient buffer system of which bicarbonate is only a minor part. It seems unlikely, therefore, with the small initial amount of bicarbonate present, that sufficiently large amounts of CO_2 have been evolved from the bicarbonate to affect materially the relative values of the two sets of R.Q. measurements. The figures obtained, i.e. an apparent R.Q. of slightly above 1 in air and of 0·5–0·6 in O_2, can therefore be taken as an indication that in high O_2 tension a large proportion of the oxidation proceeds with the formation of very little or no CO_2.

Neither cyanide nor azide affect the O_2 uptake of muscle pulp with or without addition of substrate and M.B. The respiration of whole worms is also unaffected for long periods by cyanide, while azide kills the worms within a few hours; the initial respiratory rate, however, is unaffected. Death is assumed not to be due to the poisoning of the oxidative system, but to other secondary effects.

The pattern of the oxidative enzymic system of a parasitic worm, *A. suis*, as described in this paper, seems to show a perfect adaptation to its surroundings, i.e. to the low O_2 tension; or vice versa, the low O_2 requirement may be considered as conditioned by the peculiar enzymatic system.

SUMMARY

The oxidative metabolism of a parasitic helminth, *Ascaris suis*, has been studied.

1. The O_2 uptake of muscle pulp and of whole worms is dependent on the O_2 tension, rising with increasing tension.

2. Prolonged anaerobiosis causes an O_2 debt.

3. The normal rate of oxidation is low. To this end the following factors contribute, in addition to the low O_2 tension in the intestine of the host:

(*a*) the low concentration of cytochrome in spite of the presence of succinic dehydrogenase,

(*b*) the apparent inability of the worm to deal with accumulated oxaloacetic acid, which decreases the activity of succinic dehydrogenase.

4. Increased O_2 uptake, whether caused by the addition of methylene blue or by an increase of the O_2 tension, is accompanied by the formation of H_2O_2 which is not rapidly decomposed, owing to the very low concentration of catalase.

5. Worms in an atmosphere of pure O_2 die fairly quickly with characteristic symptoms. H_2O_2 is formed and is assumed to be the cause of death.

6. In O_2 hypertension oxyhaemoglobin is oxidized by H_2O_2 to methaemoglobin. This can act as a weak peroxidase. It is thought possible that small amounts of H_2O_2, if formed at low oxidation rates and low O_2 tension, may be eliminated by secondary peroxidatic oxidation reactions catalyzed by methaemoglobin.

I wish to express my thanks to Prof. D. Keilin for many helpful suggestions during the course of these experiments.

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Studies on the Metabolism of Mould Fungi

I. PHOSPHORUS METABOLISM IN MOULDS

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Phosphorus, together with nitrogen, sulphur, magnesium and organically bound carbon, has been one of the few elements long recognized as indispensable for the growth and metabolic activities of the mould fungi (Lafar, 1904). Much later other elements, such as iron, copper, zinc, manganese, molybdenum and gallium, were added to the list of essential nutrients (Foster, 1939). It has been repeatedly shown that many moulds, including numerous species of *Aspergillus*, *Penicillium* and *Rhizopus*, are unable to grow unless small amounts of phosphate are present in the nutrient medium.

The first systematic study of the phosphorus metabolism in moulds was carried out by Vorbrodt (1920, 1926, 1928), who demonstrated that growing mycelia of *Aspergillus niger* take up orthophosphate from the culture media and store it partly in the inorganic form and partly in the form of new compounds. More recently, Michel-Durant (1938) observed that the uptake of orthophosphate by *A. niger* is insignificant at the early stage of mycelial growth but that it assumes bigger proportions at the stage of the formation of the conidia. According to this author, there are three groups of phosphorus compounds in the mycelium, a small 'lipoid fraction' and two ethanol-insoluble fractions, one of which is soluble in trichloroacetic acid ('phosphore organique soluble indéterminé') and the other acid-insoluble, represented by the non-extractable residue.

The paucity of data concerning the chemical nature of the phosphorus compounds in moulds and their metabolic function is in striking contrast to the wealth of information available regarding phosphorus compounds in yeast, bacteria, higher plants and animal tissues. It seemed, therefore, desirable to investigate the nature and behaviour of phosphorus compounds in moulds, and to examine the possibility of the existence in moulds of a link between the phosphorus metabolism and other processes, such as the metabolism of carbohydrate. (Preliminary communication, Mann, 1943.)

EXPERIMENTAL

The stock cultures used for inoculation were grown on beerwort-agar slopes and were subcultured once a month. The majority of the experiments was carried out with *A. niger* v. Tiegh. (National Collection of Type Cultures (N.C.T.C.) no. 594), but occasionally other moulds were used, such as *A. niger citricus* Neuberg (N.C.T.C. no. 1692), *A. niger* Thaysen (N.C.T.C. no. 603), *Penicillium oxalicum* Currie-Thom (N.C.T.C. no. 983) and *P. notatum* Westling-Fleming (N.C.T.C. no. 4222). The culture media were composed of 10% glucose, 0·5% $NaNO_3$, 0·1% $MgSO_4.7H_2O$, with varying amounts of K_2HPO_4, dissolved in tap water. 50–300 ml. portions of the medium were used and distributed in crystallizing dishes to a depth not exceeding 1·5 cm. The inoculum amounted to approximately 1 mg. of spore material (dry wt.) per 100 ml. medium. The mycelia were usually grown at 30° under aseptic conditions.

The disappearance of glucose from the medium of the growing culture was followed quantitatively by the method of Benedict. The total amount of organic acids produced and excreted by the mycelium into the medium was assessed by titration with 0·1 N-NaOH in presence of phenolphthalein as indicator. Owing to the presence of gluconic acid in lactone form, a rather long time was required to reach the end-point of titration. Besides gluconic acid, the two other main organic acids produced were citric acid and oxalic acid. Citric acid was determined gravimetrically as pentabromoacetone and oxalic acid as calcium oxalate.

Before performing any phosphorus analysis of the mycelium, the medium was siphoned from beneath the mycelium and replaced by the same volume of water. Care was taken not to submerge any part of the surface of the mycelium. After two more changes of water at 8 hr. intervals, the mycelium was spread evenly on a glass plate and quickly divided with a sharp knife into several parts. Some of the material was used for the estimation of the dry weight (at 105°) and for the determination of the total content of phosphorus, and the remainder was thoroughly extracted with cold trichloroacetic acid. This extract, which contained the *total acid-soluble phosphorus*, was used for the estimation of: (1) the true inorganic phosphorus determined as $MgNH_4PO_4$; (2) phosphorus determined as phosphate which reacts directly with ammonium molybdate, P_0; (3) the phosphorus which appears as orthophosphate after 7 and 30 min. hydrolysis with N-HCl, P_7 and P_{30}; (4) phosphorus as the total phosphate after incineration with H_2SO_4 and HNO_3, $P_{tot.}$ (Lohmann & Jendrassik, 1926).

Nitrogen was estimated by the Kjeldahl procedure. The assays for nicotinic acid, riboflavin and aneurin were very kindly undertaken by Dr E. Kodiček and Dr Y. L. Wang of the Nutritional Laboratory, Cambridge.

Growth and metabolism of Aspergillus niger *in presence of varying concentrations of phosphate*

With 0·002 % K_2HPO_4 or less in the culture medium no growth or only a very poor growth of *A. niger* was observed and conidia appeared very late, if at all. With 0·02 % K_2HPO_4, thin mycelia appeared 2 days after sowing and developed within the next 2 days into strong, white, smooth pellicles. Usually on the 4th day of growth the first black conidiophores made their appearance. With 0·2–0·5 % K_2HPO_4 the growth was still quicker, and within 4 days a very thick mycelium was produced which, instead of being white and smooth, showed a creased surface and was of a distinctly yellowish colour, especially at the surface in contact with the medium. No conidiophores were present at that stage. They usually appeared on the 6th or 7th day of growth.

Both the rate at which phosphate was absorbed from the medium as well as the concentration which it finally reached in the mycelium were found to depend on the initial amount of K_2HPO_4 in the medium. With 0·02 % K_2HPO_4 in the medium, the rate of disappearance was so rapid that after 3 days of growth the medium was completely depleted of phosphorus; the final concentration of phosphorus reached in the mycelium was about 0·3 % P (dry wt.). With 0·2–0·5 % K_2HPO_4 in the medium, the rate of absorption of phosphate by the growing mycelium was somewhat slower during the first days of growth and reached a higher level only at the preconidial stage; the final concentration of phosphorus in the mycelium was 1–2 % P (dry wt.).

Effect of phosphate concentration on metabolism. By varying the concentration of phosphate in the medium it was possible to influence markedly both the carbohydrate and the nitrogen metabolism of the mould, as well as its respiration. Table 1 illustrates the differences between two cultures, A and B, grown in the presence of 0·02 and 0·2 % K_2HPO_4, respectively. The culture B grew faster than the culture A; it utilized glucose more rapidly and turned a higher proportion of it into citric acid. In addition, it was capable of building up a much larger store of nitrogen than the phosphorus-deficient culture A. The nitrogen stored in the mycelium, derived from the nitrate of the medium, was found to be entirely of organic nature; neither nitrate nor free ammonia could be detected. Approximately one-half of the mycelium-N could be extracted with trichloroacetic acid or with other deproteinizing reagents. Among the N-containing substances thus extracted were aneurin, riboflavin and nicotinic acid. It is of considerable interest to note that in response to an increased concentration of inorganic phosphate in the medium, the formation of all three vitamins was enhanced to such an extent as to compare favourably with rich vitamin sources such as liver or yeast. The high riboflavin content is responsible for the yellow colour of the phosphorus-rich mycelium, and also, it may have some bearing on the fact that such a mycelium, when separated from its medium and transferred to water, continued to respire for several days much more vigorously

Table 1. *Effect of varying concentration of orthophosphate on the metabolism of* A. niger

	A grown in presence of 0·02 % K_2HPO_4	B grown in presence of 0·2 % K_2HPO_4
Mycelium (5 days old)		
Appearance	Thin, white, smooth, first conidiophores	Thick, yellowish, no conidiophores
Dry weight	460 mg.	1092 mg.
Q_{O_2} of intact mycelium	6·2	11·4
Q_{O_2} of mycelium pulp	0·4	1·9
Q_{O_2} of pulp + 1 % glucose	30·0	19·0
Total nitrogen, mg.	8·1	23·7
Total phosphorus, mg.	1·5	12·1
Magnesium, mg.	3·8	6·8
Aneurin, μg.	3·2	19·0
Riboflavin, μg.	16·1	78·7
Nicotinic acid, μg.	19·4	302·0
Medium (50 ml.)		
Appearance	Colourless	Yellow
Glucose, %	3·7	0·1
Glucose oxidase activity; 2 ml. medium + 10 mg. glucose, pH 7, 30°, take up in 15 min.	120 μl. O_2	30 μl. O_2
Total phosphorus, mg.	0·0	6·3
Titratable total acidity, ml. N-acid	15·0	16·0
Citric acid, ml. N-acid	3·8	7·9
Oxalic acid, ml. N-acid	0·05	0·0

than the P-deficient mycelium. Grinding of the mycelia with water caused a pronounced fall in the O_2 uptake, but even then, the pulp obtained from the mycelium B had a much higher rate of O_2 uptake than that prepared from mycelium A. When, however, glucose was added to the pulp, the O_2 uptake increased more in A than in B. This was due to a higher activity of glucose oxidase in the mycelium A.

The absorption of phosphate from the medium and the formation of phosphorus compounds in the mycelium

Absorption of phosphate. The experiments recorded in Table 2 were carried out with *A. niger* v. Tiegh. in crystallizing dishes containing 50 ml. of the medium described, with 0·2 % K_2HPO_4 and grown at 30°. Thin mycelia usually appeared 24 hr. after sowing, and the production of gluconic and citric acid, at the expense of glucose, soon followed. At the same time, the absorption of phosphate from the medium began to be noticeable. Occasionally the rate of the absorption of phosphate by the growing mycelium was rather slow during the first days of growth and reached a higher velocity only at the preconidial stage. In other instances, however, the absorption proceeded rapidly from the beginning of the growth and was well marked at an early stage of growth, several days before the appearance of the first conidiophores.

Formation of phosphorus compounds in the mycelia. In order to follow the fate of the absorbed phosphate, a study was made of the content and the distribution of phosphorus compounds in the mycelia. A large proportion of the phosphorus which accumulated in the mycelia, sometimes as much as 90 %, could be extracted by grinding the mycelia thoroughly with trichloroacetic acid. Only a small proportion of the acid-soluble phosphorus, 10–20 % in all, behaved like ordinary orthophosphate, and the value of the phosphorus determined directly (P_0) equalled that in the magnesium-ammonium-phosphate precipitate. A large proportion of the total acid-soluble phosphorus, as assessed by incineration with H_2SO_4 and HNO_3, broke down completely to orthophosphate after a 7 min. hydrolysis with 1 N-HCl at 100°, and thus behaved like a typical 'easily acid-hydrolyzable phosphorus compound'. Only a small proportion of the phosphorus present in the mycelium was found to occur in a 'difficultly hydrolyzable' form.

The formation of the phosphorus compounds in the mycelium usually reached its peak at the time when the first conidiophores were appearing. At the same time the medium was already depleted of 50–90 % of the orthophosphate originally present, as well as of glucose, and contained large quantities of gluconic acid and citric acid. When the concentration of these two acids had reached its maximum, oxalic acid was still absent, and could be found only later, after the mycelium had lived for several days at the expense of the first two organic acids. As soon as the reserve of gluconic and citric acid was exhausted, the process of autolysis set in, heralded by a decrease in the dry weight of the mycelium and accompanied by a rapid decomposition of the phosphorus compounds in the mycelium and the reappearance of orthophosphate in the medium.

Table 2. *Phosphorus metabolism in growing cultures of* A. niger

(P_0 = phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_7 = the phosphorus which appears as orthophosphate after 7 min. hydrolysis with N-HCl; P_{30} = the phosphorus which appears as orthophosphate after 30 min. hydrolysis with N-HCl; $P_{tot.}$ = phosphorus as the total phosphate after incineration with H_2SO_4 and HNO_3.)

			Mycelium					Medium (50 ml.)					
				Trichloroacetic acid extracts mg. P									
	Age (days)	Appearance	Dry wt. (g.)	P_0	P_7	P_{30}	$P_{tot.}$	Glucose (g.)	Total acidity (ml. N-acid)	P_0 (mg.)	$P_{tot.}$ (mg.)	Citric acid (ml. N-acid)	Oxalic acid (ml. N-acid)
	0		—	—	—	—	—	5·0	0·0	17·8	17·8	—	—
I	2	Thin, continuous	0·506	0·4	1·6	—	—	—	3·2	—	—	0·6	0·0
	3	White, strong	1·056	1·5	5·0	5·5	6·5	1·1	15·0	10·5	10·5	3·9	0·0
	5	First conidia	1·340	1·5	9·0	9·5	11·0	0·1	18·0	5·0	5·0	6·9	0·0
	9	Many conidia	1·728	1·1	12·3	12·9	14·0	0·0	5·5	0·0	0·0	5·5	0·1
	14	Autolysis	0·932	2·6	4·8	—	—	0·0	0·3	8·0	9·6	0·0	0·3
II	2	Thin, continuous	0·352	0·2	1·5	1·5	1·8	1·1	6·5	16·0	16·0	—	—
	4	White, strong	0·760	0·7	5·7	6·1	10·0	0·3	12·5	—	—	—	—
	6	Few conidia	1·356	2·3	9·0	10·3	12·0	0·0	7·5	0·0	0·0	—	—
	14	Autolysis	0·816	4·6	6·3	6·3	6·8	0·0	0·0	6·0	6·0	—	—
III	3	White, strong	0·320	1·5	5·0	—	—	1·1	13·0	—	—	4·0	0·0
	5	First conidia	0·792	0·7	8·6	—	—	0·0	15·5	—	—	4·6	0·0
	8	Many conidia	1·150	0·6	10·0	—	12·0	0·0	8·0	0·0	0·0	5·0	0·0
	12	Onset of autolysis	0·920	—	—	—	—	0·0	2·0	0·3	0·5	1·0	1·0
	19	Autolysis	0·510	—	—	—	—	0·0	0·0	11·0	12·0	0·0	Trace

Table 3. *Formation of an easily acid-hydrolyzable phosphorus compound in moulds*

Species	Mycelium: Dry wt. (g.)	Mycelium: Trichloroacetic acid extracts (mg. P) *P_0	Mycelium: Trichloroacetic acid extracts (mg. P) *P_7	Medium (50 ml.): Glucose (g.)	Medium (50 ml.): Acidity (ml. N-acid)	Medium (50 ml.): *P_0 (mg.)
A. niger v. Tiegh.	1·196	2·7	9·4	0·0	9·5	2·5
A. niger citricus Neuberg	1·008	0·6	1·3	0·8	11·5	12·5
A. niger Thaysen	1·252	5·3	6·6	0·0	1·5	2·5
P. oxalicum	0·624	0·2	3·0	3·3	0·75	10·0
P. notatum	0·960	1·3	4·7	Trace	10·5	10·0

* See note to Table 2.

The formation of easily hydrolyzable phosphorus from orthophosphate is not restricted to one strain of *A. niger* but was found also with other moulds (Table 3). However, the amounts of the readily hydrolyzable phosphorus formed by these moulds were smaller than with *A. niger* v. Tiegh.

Effect of oxygen and respiratory inhibitors on phosphorus metabolism. The absorption of phosphate from the medium and the formation of phosphorus compounds in the mycelium were found to depend strictly on the presence of oxygen. In this respect the phosphorus metabolism of moulds differs substantially from the various phosphorylation processes which occur in yeast and in animal tissues. Cyanide and azide (0·001 N), iodoacetate (0·001 N) and fluoride (0·005 N) were found to have a powerful inhibitory effect on the respiration as well as on the carbohydrate metabolism of the moulds; at the same time these inhibitors strongly suppressed the phosphorus metabolism.

The effect of these inhibitors could best be studied on mycelia grown directly in the cups of Barcroft differential manometers (Table 4).

A. niger was sown on 5 ml. medium. During the early stages of growth the shaking of the manometers had to be avoided owing to the great fragility of the fresh mycelium. With a continuous mycelium gentle shaking could be safely applied, but it had little effect on the extent of the oxygen consumption owing to the fact that the mycelium respires mainly on the surface. After 4 days of growth at 30° the manometer cups containing the pellicles were attached to the manometers and the course of the respiration was followed quantitatively. The oxygen uptake of the mycelia was measured at 30°, and expressed in terms of

$$Q_{O_2} = \frac{\mu l.\ O_2 \text{ taken up}}{\text{mg. dry wt.} \times \text{hr.}}.$$

On the addition of inhibitors the absorption of phosphate from the medium came suddenly to a standstill. The action of the inhibitors on the respiration and the phosphate absorption could be reversed provided the mycelium had not been left in contact with the poison for more than a few hours. Otherwise an autolysis set in and the phosphorus compounds formed in the mycelium broke down rapidly with the liberation of free phosphate.

In order to demonstrate the effect of oxygen on the phosphate metabolism it was found convenient to grow mycelia for several days on media deficient in phosphate (0·01–0·02 % instead of 0·2 % K_2HPO_4), but otherwise composed as described above. If these mycelia were then transferred to 0·1 % KH_2PO_4 in

Table 4. *Effect of respiratory inhibitors on the phosphorus metabolism*

Mycelium grown on 5 ml. medium in Barcroft manometer cups; 4 days at 30°.

Addition to the medium: Substance	Concentration (M)	Analyzed after (hr.)	Mycelium: Dry wt. (mg.)	Mycelium: Q_{O_2}	Mycelium: *P_0 (mg.)	Mycelium: *P_7 (mg.)	Medium: *P_0 (mg.)	Medium: *P_7 (mg.)
—	—	—	90	12·7	0·07	0·60	0·08	0·08
Iodoacetate	0·001	3	90	3·6	0·09	0·60	0·09	0·09
	0·001	24	82	1·6	0·09	0·40	0·30	0·32
Fluoride	0·005	3	—	5·8	—	—	—	—
	0·005	24	80	2·3	0·09	0·40	0·10	0·10
	0·01	3	—	4·8	—	—	—	—
	0·01	24	80	1·9	0·05	0·40	0·20	0·25
	0·02	3	—	1·3	—	—	—	—
	0·02	24	60	0·5	0·05	0·40	0·20	0·25
Azide	0·001	3	—	2·8	0·07	0·50	0·10	0·10
	0·001	24	65	1·3	0·09	0·40	0·30	0·32
Cyanide	0·001	3	90	1·5	0·08	0·55	0·10	0·10

* See note to Table 2.

the presence of oxygen, they quickly utilized the inorganic phosphate and built up from it the easily hydrolyzable phosphorus compound (Fig. 1, curve I). Anaerobically, there was no change in the content of KH_2PO_4 in the medium (Fig. 1, curve II). When, after a period of anaerobic incubation, oxygen was let in, the mycelium responded immediately with renewed absorption of phosphate. However, the absorption never reached the same level as with mycelia which had not been exposed to anaerobic conditions. The reason is that even a short period of anaerobiosis has a general adverse effect on the metabolic activities of the mycelium. A prolonged lack of oxygen was found to lead invariably to autolysis.

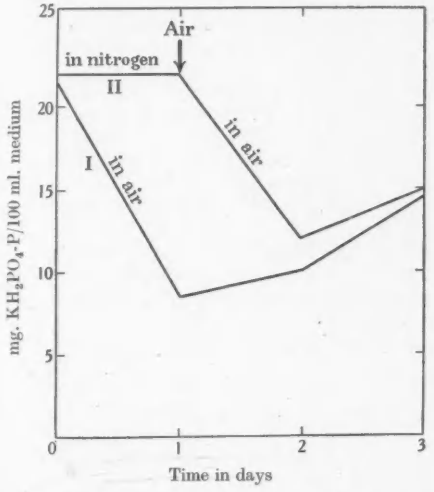


Fig. 1. The absorption of phosphate from the medium in presence and absence of oxygen

When mycelia were transferred to neutralized solutions of sodium pyrophosphate or of certain sugar-phosphoric acid esters instead of to orthophosphate, the process of phosphate absorption was slower because it was preceded by the breakdown of the phosphoric compounds into orthophosphate (Table 5). Glucose had a slightly delaying effect on the process of phosphate absorption.

Metaphosphatase

It is essential to use trichloroacetic acid for the successful extraction of phosphorus compounds from the mycelium. When the mycelium was ground with water instead of with trichloroacetic acid, the acid-hydrolyzable phosphorus compounds of the pulp broke down within a short time to orthophosphate. They could be preserved, however, either by heating the pulp to 100° and keeping it at this temperature for a few minutes or by rapidly dehydrating the pulp with acetone and converting to a dry powder. No losses in the acid-soluble phosphorus occurred during the acetone treatment which, however, did remove all acetone-soluble substances including some acetone-soluble, difficultly hydrolyzable phosphorus compound. The distribution of phosphorus, nitrogen and magnesium, between the acetone extract, the acetone powder and the trichloroacetic acid extract from the acetone powder of one mycelium, is illustrated by the scheme shown on p. 344.

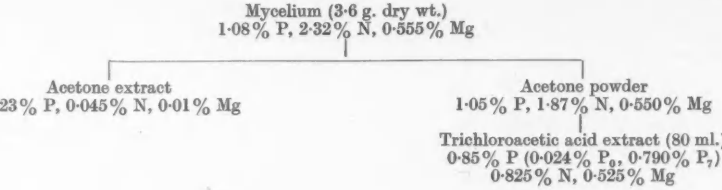
The assumption that the breakdown of phosphorus compounds in the mycelium ground with water might be due to the presence of a phosphatase was strengthened by the observation that if such a pulp was brought into contact with a variety of phosphoric acid derivatives, it decomposed them quickly into orthophosphate.

The phosphatase contained in the mycelium could be extracted with water without any loss of activity. The extract (1 ml.; 12 mg. dry wt.), when acting upon 2 ml. substrate (Na salts; 1·2 mg. P) for 3 hr. at pH 5 and 30°, converted to orthophosphate 70–98 % of the following compounds: pyrophosphate, metaphosphate, hexametaphosphate, α-phosphoglycerol, β-phosphoglycerol, Cori ester (1-phosphoglucose), Embden ester (6-phospho-hexose),

Table 5. *Metabolism of pyrophosphate and sugar-phosphoric acid esters by the mycelium*

Mycelium grown in manometer vessel. On the fifth day medium replaced by 5 ml. of:	Incubation at 30° (hr.)	Q_{O_2} of mycelium	Media		
			*P_0 (mg.)	*P_7 (mg.)	Acidity (ml. N-acid)
Water	0	8·3	0·0	0·0	0·0
	16	6·3	0·05	0·05	0·25
Sodium pyrophosphate (4 mg. P)	0	9·2	0·0	4·0	0·0
	16	10·0	2·5	3·0	0·14
K salt of Cori ester (6 mg. P)	0	9·4	0·0	6·0	0·0
	16	12·8	3·9	4·5	0·16
Na salt of Embden ester (6 mg. P)	0	8·6	0·0	0·1	0·0
	16	11·9	2·5	2·5	0·08

* See note to Table 2.


Mycelium (3·6 g. dry wt.)
1·08% P, 2·32% N, 0·555% Mg
Acetone extract
0·023% P, 0·045% N, 0·01% Mg
Acetone powder
1·05% P, 1·87% N, 0·550% Mg
Trichloroacetic acid extract (80 ml.)
0·85% P (0·024% P_a, 0·790% P_i)
0·825% N, 0·525% Mg

Harden-Young ester (1:6-diphospho-fructose), 3-phosphoglycerate, adenosine triphosphate (all three atoms of phosphorus), muscle adenylate, yeast adenylate, aneurin diphosphate and phytate.

The outstanding property of this polyphosphatase, and one which sets it apart from other known phosphatases, is the ease with which it acted on metaphosphate. This enzyme may therefore be referred to as a *metaphosphatase*.

The only instance where metaphosphatase activity has been previously described in moulds is the observation made by Kitasato (1928 *a*, *b*), who found that preparations of 'Taka-phosphatase' from *A. oryzae* are active towards metaphosphate. He had to use, however, as much as 1 g. of Taka-phosphatase and incubate this preparation for 20 hr. with 250 mg. sodium metaphosphate in order to obtain 10% of the phosphorus in the form of orthophosphate. Compared with the metaphosphatase of *A. niger* the activity of 'Taka-phosphatase' appears to be negligible.

Metaphosphatase in metabolism fluid. The examination of the culture media separated from the mycelia of *A. niger* revealed that they too possessed considerable metaphosphatase activity; 2 ml. medium separated from a 4-day-old mycelium and incubated for 5 hr. with 2 ml. sodium metaphosphate (0·6 mg. P) converted it completely to orthophosphate. Incidentally, the presence of the phosphatase in the medium provides an explanation for the observation that organic phosphorus compounds added to the culture media are first dephosphorylated and then absorbed as orthophosphate.

Purification of metaphosphatase. In order to purify the metaphosphatase and to obtain the enzyme free from glucose oxidase (always present alike in the mycelium and the medium), the aqueous extracts from the mycelia were dialyzed, the enzyme was precipitated with 3 vol. of cold acetone, redissolved in water and finally purified by adsorption on tricalcium phosphate gel and alumina C_γ. In this manner it was possible to obtain metaphosphatase preparations practically free from glucose oxidase. In addition, it was found that 0·01 M-sodium nitrate had a strong inhibitory effect on the activity of glucose oxidase at pH 5 (but not at pH 7), whereas it did not affect the activity of metaphosphatase at all. The optimum activity of metaphosphatase occurred at pH 3·7–4·2. A purified enzyme preparation (60 μg.) incubated with a solution of sodium metaphosphate or pyrophosphate (0·3 mg. P) for 2 hr. at pH 5 and 30° converted both compounds quantitatively to orthophosphate.

DISCUSSION

Unlike the animal tissues and yeast cells, the metabolic activities of the mould fungi, especially those of the acid-producing species like *Aspergillus* and *Penicillium*, depend to a large extent on the presence of oxygen. *Aspergillus niger*, for instance, will oxidize sugar very efficiently in the presence of oxygen to non-volatile organic acids such as gluconic, citric and oxalic acid, whereas anaerobically it scarcely ferments sugar at all. This phenomenon was well known to the early investigators, and Duclaux in his *Traité de Microbiologie* (1898) very appropriately referred to the process of oxalic acid formation in moulds as 'respiration oxalique', a term which is more suitable than the later 'oxalic fermentation' or 'oxidative fermentation'. The experiments described in this paper demonstrate for the first time that in addition to the carbohydrate metabolism, the phosphorus metabolism of *A. niger* is also strictly aerobic in character. In this respect the moulds differ again from yeast, where the presence or absence of oxygen is said to be of little importance so far as the absorption of phosphate is concerned (Mullins, 1942). The intimate relationship between the phosphorus metabolism and the respiration of *A. niger* is best demonstrated by the fact that various inhibitors of mould respiration, such as cyanide, azide, iodoacetate and fluoride, also stop phosphorus metabolism. This fact indicates a connexion between the metabolism of carbohydrate and that of phosphorus compounds in moulds. Moreover, an increase in the content of phosphate in the culture medium enhances both the utilization of carbohydrate and the formation of organic acids, particularly citric acid. However, the increased production of citric acid may also be due to changes in the nitrogen metabolism.

Mycelia grown in the presence of high phosphate concentrations have a much higher nitrogen metabolism than those grown on phosphate-deficient media and are capable of building up a considerable reserve of various valuable nitrogenous substances.

Another point which emerged is that the mycelia of various moulds convert a large proportion of the absorbed orthophosphate into acid-hydrolyzable phosphorus compounds. One of these compounds breaks down completely to orthophosphate after a 7 min. hydrolysis with N-HCl at 100°, and thus behaves like pyrophosphate. Another compound occurs in the 'difficultly hydrolyzable' fraction.

It is interesting to note that *A. niger*, which contains an abundant store of phosphorus compounds in the mycelium, also possesses a highly active phosphatase. This enzyme acts optimally under the acid conditions prevailing in the mould culture. It attacks a variety of derivatives of phosphoric acid, among them metaphosphate, a fact which will be commented upon in the next paper of this series.

SUMMARY

1. Mould fungi with a predominantly aerobic carbohydrate metabolism have also a characteristic aerobic phosphorus metabolism. They absorb orthophosphate aerobically and convert it into a number of phosphorus compounds.

2. The rate with which phosphate is absorbed and utilized depends on its initial concentration in the culture medium. Cultures grown in the presence of large concentrations of phosphate develop more rapidly than those grown in low concentrations, have a higher respiratory quotient, utilize glucose more rapidly, produce more citric acid, have a higher nitrogen metabolism and exhibit a substantially increased content of certain vitamins.

3. Cyanide and azide (0·001N), iodoacetate (0·001N) and fluoride (0·005N) strongly inhibit the respiration of these moulds, and bring the phosphorus metabolism to a standstill.

4. A large proportion of the orthophosphate absorbed aerobically is converted to acid-hydrolyzable compounds. One of these compounds breaks down to orthophosphate after a 7 min. hydrolysis with N-HCl, and thus behaves like a typical 'easily hydrolyzable phosphorus compound'. Another compound occurs in a 'difficultly hydrolyzable' form.

5. The formation of phosphorus compounds in the mycelium reaches its peak at the preconidial stage. During autolysis these compounds are decomposed and excreted into the medium as orthophosphate.

6. The occurrence of a metaphosphatase in both the mycelium and the culture medium of *Aspergillus niger* is described. The enzyme has been purified and separated from glucose oxidase. Whereas the activity of glucose oxidase is considerably reduced by 0·01N-$NaNO_3$, the phosphatase activity remains intact.

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Studies on the Metabolism of Mould Fungi

2. ISOLATION OF PYROPHOSPHATE AND METAPHOSPHATE FROM *ASPERGILLUS NIGER*

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The mycelium of *Aspergillus niger* contains a large proportion of its phosphorus in the form of compounds which can be easily extracted with trichloroacetic acid and which yield orthophosphoric acid after acid hydrolysis. In order to obtain more definite information with regard to the chemical nature of these compounds an attempt was made to isolate them from the mycelium.

EXPERIMENTAL

The first step in the purification procedure was the extraction of the mycelia with trichloroacetic acid. This was followed by precipitation with $Pb(NO_3)_2$, by means of which two distinct fractions were obtained which will be referred to as 'Pb *fraction I*' and 'Pb *fraction II*'. The first fraction precipitated

at pH 1·8 and was entirely composed of phosphorus compounds hydrolyzable in 7 min. by N-HCl to orthophosphate. By means of $Ba(NO_3)_2$ two compounds were obtained from this fraction and identified as Ba pyrophosphate and Ba metaphosphate, respectively. The 'Pb fraction II', which precipitated at pH 7, was a mixture containing orthophosphate, some easily hydrolyzable phosphate and a high proportion of a difficultly hydrolyzable phosphorus compound; the latter has been separated from the rest as a water-soluble Ba salt.

Several derivatives were prepared of the phosphorus compounds isolated from *A. niger*. The barium derivatives were analyzed by Dr Weiler (Oxford) for their Ba and C content. In the other derivatives Cu was determined as diethyl-dithiocarbamate, Pb and Zn as compounds with dithizone, Ca as Ca oxalate, and Mg as $MgNH_4PO_4$.

To distinguish between metaphosphate and pyrophosphate use was also made of several enzymic methods. The following enzymes were used: (1) succinic oxidase, prepared from horse heart muscle by the method of Keilin & Hartree (1938); (2) pyrophosphatase, a preparation obtained from yeast and kindly given me by Drs Bailey, Dixon and Webb; and (3) metaphosphatase prepared from *A. niger*.

Below, details are given of the methods employed for the isolation of the phosphorus compounds.

Separation of phosphorus compounds as lead and barium salts

A. niger v. Tiegh. was grown at 28° on a medium composed of 10% glucose, 0·2% K_2HPO_4, 0·4% $NaNO_3$ and 0·05% $MgSO_4.7H_2O$. Cultivation was carried out in crystallizing dishes containing 200 ml. medium. This produced a continuous mycelium within 2 days, and from the third day onwards the medium was strongly acid (pH 2–2·5). It was found that mycelia grown under aseptic conditions and those grown without any special precautions had the same content and the same distribution of phosphorus compounds. In view of this fact, the large-scale experiments were carried out under non-aseptic conditions.

Nine 9-day-old mycelia (fresh wt. 270 g., dry wt. 41 g.) were ground with sand and 700 ml. cold 10% trichloroacetic acid in a mechanical mortar for 15 min. After standing for 2 hr. at 0°, the extract was centrifuged. The residue was mixed with 400 ml. 5% trichloroacetic acid and centrifuged after standing at 0° overnight. Both extractions yielded together 1100 ml. of a turbid yellowish fluid. The phosphorus content of the extract was 0·038 mg. P_0, 0·191 mg. P_7, 0·212 mg. P_{30} and 0·279 mg. $P_{tot.}$ per ml. (where P_0 = phosphorus as phosphate which reacts directly with ammonium molybdate; P_7 and P_{30} = phosphorus as phosphate which appears as orthophosphate after 7 min. and after 30 min. hydrolysis with N-HCl, respectively; $P_{tot.}$ = total phosphorus after incineration with H_2SO_4 and HNO_3 (Lohmann & Jendrassik, 1926; see Mann, 1944). The extract also contained 0·38 mg. N and 0·04 mg. pentose per ml.

The extract (1 l.) was brought to pH 1·8 with NaOH and treated with 30 ml. saturated solution of $Pb(NO_3)_2$. The white precipitate, washed and decomposed with H_2S, yielded 450 ml. of a strongly opalescent filtrate, the easily hydrolyzable 'Pb fraction I' containing 0·002 mg. P_0, 0·234 mg. P_7, and 0·244 mg. $P_{tot.}$ per ml. The clear, strongly yellow-coloured supernatant fluid separated by centrifuging from the first lead precipitate was neutralized with NaOH. The resulting precipitate was left at 0° overnight, centrifuged, washed with water and decomposed with H_2S. It yielded 470 ml. of a yellow-coloured solution, the 'Pb fraction II' containing 0·112 mg. P_0, 0·234 mg. P_7, 0·264 mg. P_{30} and 0·435 mg. $P_{tot.}$ per ml., and distinguished from the 'Pb fraction I' by a high content of a difficultly hydrolyzable phosphorus compound.

Before subjecting the 'Pb fraction I' to a fractionation with barium nitrate, it was purified by reprecipitating with $Pb(NO_3)_2$ at pH 2 and removing Pb with H_2S.

The solution of 'Pb fraction I' thus obtained was completely free from trichloroacetic acid and showed distinctly the ability to precipitate protein. This was tested in the following way: 1 ml. of 1:20 diluted horse serum was acidified with a drop of N-acetic acid and followed by a drop of the neutralized solution of the 'Pb fraction I'. Immediately the serum protein precipitated.

400 ml. of the 'Pb fraction I' was treated at pH 2 with 10 ml. of saturated $Ba(NO_3)_2$, left for 1 hr. and centrifuged.

Table 1. *Separation of phosphorus compounds from mycelia of* A. niger *grown in presence of low and high phosphate concentration*

(P_0 = mg. phosphorus as phosphate which reacts directly with ammonium molybdate; P_7 = mg. phosphorus as orthophosphate after 7 min. hydrolysis with N-HCl; P_{30} = mg. phosphorus as orthophosphate after 30 min. hydrolysis with N-HCl; $P_{tot.}$ = mg. phosphorus as phosphate after incineration with H_2SO_4 and HNO_3.)

	Amount of the different compounds (mg.) obtained from mycelium grown on 200 ml. of culture medium									
	A. Low phosphate medium					B. High phosphate medium				
	P_0	P_7	P_{30}	$P_{tot.}$	N	P_0	P_7	P_{30}	$P_{tot.}$	N
Culture medium only	7·1	0·0	0·0	0·0	65·0	71·0	0·0	0·0	0·0	65·0
Trichloroacetic extract from one mycelium	Trace	2·7	3·1	4·8	16·6	6·4	19·2	21·2	33·1	28·3
'Pb fraction I'	0·4	1·3	1·4	1·5	0·4	4·2	10·6	11·4	12·2	0·8
'Ba metaphosphate fraction'	0·0	0·5	0·5	0·5	—	0·2	3·8	3·9	4·0	—
'Ba pyrophosphate fraction'	0·3	0·7	0·7	0·8	—	0·8	4·1	4·1	4·7	—
'Pb fraction II'	0·0	0·0	0·3	3·8	—	0·0	Trace	0·5	7·2	2·9

The supernatant fluid, devoid of the protein-precipitating compound, was brought to pH 5 with NaOH. This produced a second precipitate.

Both barium precipitates, referred to below as the 'Ba *metaphosphate fraction*' and 'Ba *pyrophosphate fraction*', were washed several times with cold water, finally with ethanol, and dried in vacuum over $CaCl_2$. In this manner two colourless products were obtained: 'Ba metaphosphate fraction' 420 mg., 9·5% P; 'Ba pyrophosphate fraction' 260 mg., 12·5% P. Both substances were free from orthophosphate, and were completely hydrolyzed by N-HCl in 7 min. Similar compounds were obtained from a mycelium grown under conditions where little phosphate (0·02% K_2HPO_4 instead of 0·2% K_2HPO_4) was available to the growing culture. The yields were, however, correspondingly smaller (Table 1).

Isolation of pyrophosphate

The 'Ba-pyrophosphate fraction' was suspended in water acidified with acetic acid and centrifuged. The centrifuged precipitate was dissolved in dilute HCl, and Ba removed with N-H_2SO_4. The Ba-free solution was then neutralized with NaOH and the reaction finally adjusted to pH 4·5. On addition of $CuSO_4$ a slight opalescence appeared which gradually increased and turned into a fine, microcrystalline, pale blue precipitate. After 24 hr. at 0°, this was centrifuged, washed repeatedly with water, and finally dried in vacuum over $CaCl_2$. This yielded 105 mg. of a pale blue-coloured substance, 3 mg. of which were dissolved in 9 ml. 0·02 N-HCl and analyzed for its Cu and P content. 57 and 112 μg. Cu were found in 0·5 and 1 ml., respectively. The phosphate analysis was carried out in three 2 ml. samples; found: P_0, 0 μg.; P_7, 106 μg.; $P_{tot.}$, 108 μg.

The atomic ratio Cu : P calculated from the above figures was 1 Cu/0·96 P as compared with the theoretical ratio of 1 Cu/1 P in $Cu_2P_2O_7$. The high water content of the preparation could be only partly reduced by drying at 100°. Even at 110° the preparation still retained about 2 mol. water/1 mol. $Cu_2P_2O_7$.

From the Cu pyrophosphate other derivatives were obtained; 20 mg. samples were used for the preparation of Ag, Zn and Cd pyrophosphates. From the remaining copper salt crystalline Na pyrophosphate was prepared:

The Cu salt (35 mg.) was suspended in 20 ml. water, Cu removed with H_2S, the filtrate aerated and carefully treated with diluted NaOH until it became distinctly red to phenolphthalein but only slightly blue to thymol blue. The water-clear solution was left in a desiccator over $CaCl_2$ for several days. On the third day clusters of large monoclinic crystals appeared. A sample of the crystalline material was dried and 2 mg. of the dry material analyzed; it contained 22·8% pyrophosphate-P and only a trace of orthophosphate.

Isolation of metaphosphate

The 'Ba metaphosphate fraction' obtained by precipitation with $Ba(NO_3)_2$ at pH 2 was extracted three times with 20 ml. cold 0·5 N-H_2SO_4, and the combined extracts neutralized. The colourless, water-clear solution thus obtained still had some protein-precipitating activity although much less than the solution from which the 'Ba metaphosphate fraction' had originally been prepared.

The solution was precipitated with 5 ml. 10% $AgNO_3$, the pH adjusted to 5, and the precipitate left at 0°. Next day, the precipitated Ag compound was centrifuged, washed with water, and decomposed with H_2S.

Following this treatment, however, the solution had lost its protein-precipitating property. On addition of $Ba(OH)_2$ to the point where the solution became just pink to phenolphthalein a precipitate was obtained. This gave, after washing and drying, 70 mg. of a substance containing 63·4% Ba and 14·9% P, of which 13·9% was hydrolyzable in 7 min., the rest being orthophosphate. By comparing these figures with the analytical data for pure pyrophosphate, $Ba_2P_2O_7$, 59·8% Ba and 13·8% P, and pure metaphosphate, $Ba(PO_3)_2$, 46·4% Ba and 21% P, it was obvious that the purification of the 'Ba metaphosphate fraction' had resulted in the formation of a Ba compound identical with Ba pyrophosphate but not with Ba metaphosphate. This, together with the observation that the purification procedure brought about a complete loss of the protein-precipitating property, led to the suspicion that the phosphorus compound, originally present in the 'Ba metaphosphate fraction', might be a polymetaphosphoric acid which easily underwent hydrolysis to pyrophosphoric acid. To test this, a new method was designed by which it was possible to isolate the mould-metaphosphate in the native state as its water-soluble Na and NH_4 salts. This was carried out in the following manner.

Eight 9-day-old mycelia were extracted with cold 10% trichloroacetic acid. The extract (1 l.) was treated with ethanol (1 l.), left at 0° for several hours, and the flocculent precipitate removed by centrifugation and filtration. On addition of NaOH to the clear yellow filtrate, there appeared at pH 5·5 a precipitate which was centrifuged 24 hr. later and dried over $CaCl_2$. It yielded 1 g. of a white substance which was free from orthophosphate but contained 13% of phosphorus hydrolyzable in 7 min. in N-HCl. When, instead of NaOH, ammonia was used for the neutralization, the yield of the easily hydrolyzable phosphorus compound was the same but its P content was 16%. The crude preparations of the Na and NH_4 salts were found to contain Ca and Mg, probably in close association with a part of the easily hydrolyzable P. Thus in 100 mg. of the ammonium salt were found: P, 16 mg.; NH_4-N, 6 mg.; Ca, 5 mg.; Mg, 4·7 mg.; Zn, 0·3 mg.; Fe, 0·1 mg. It also contained 0·24 mg. pentose.

The above preparations always contained some water-insoluble residue, amounting to 5–25% of the easily hydrolyzable P, associated with Ca and Mg but not with Na or NH_4. In the course of this

work several preparations of the crude sodium and ammonium salts were made. The aqueous solutions of these preparations varied in pH from 5·8 to 7·6, but they invariably exhibited the protein-precipitating property, a distinct positive test being obtained with about 0·3 mg.

Purification of ammonium and sodium salts. In order to purify the crude sodium and ammonium salts, 300 mg. of each product were dissolved separately in 100 ml. water, the aqueous solutions treated with 100 ml. 10% trichloroacetic acid and 200 ml. ethanol, left overnight at 0°, filtered and the filtrates neutralized with NH_3 or NaOH to methyl red. On standing at 0° the ammonium and sodium salts came down. They contained 23 and 20% P respectively. At this stage the atomic ratio P_7:NH_4 (or Na) was 1:1, as could be expected from a compound built on the model of a metaphosphate, NH_4PO_3 or $NaPO_3$, or a polymetaphosphate, $(NH_4PO_3)_n$ or $(NaPO_3)_n$. On the other hand, there was the possibility that the compound purified in such a manner might be an acid pyrophosphate, $(NH_4)_2H_2P_2O_7$ or $Na_2H_2P_2O_7$, in which case also the ratio P_7:NH_4 (or Na) would have been 1:1. To test this possibility the following experiment was performed.

Five aqueous solutions were prepared: (1) NH_4 salt obtained from the mould, (2) Na salt obtained from the mould, (3) crystalline Na pyrophosphate, (4) Na metaphosphate, and (5) Na hexametaphosphate. All five solutions, each containing exactly 3 mg. P in 5 ml., were treated in centrifuge tubes with 5 ml. 10% trichloroacetic acid and 10 ml. ethanol, filtered clear, neutralized with NaOH to methyl red and left at 0° for 24 hr. There was a precipitate in all tubes except tube 3 which had pyrophosphate, and in which the solution remained perfectly clear. In the centrifuged precipitates the following quantities of P_7 were recovered: (1) 2·4 mg., (2) 2·3 mg., (4) 2·7 mg., and (5) 2·9 mg.

This experiment showed that acid pyrophosphate is not precipitated under these conditions, and also revealed a convenient method by which pyrophosphoric acid can be distinguished from metaphosphoric acid. The lower yields in the samples (1) and (2) were due to the fact that the preparations of the Na and NH_4 salts from the mould still contained some pyrophosphate.

On repeating several times the acid-ethanol treatment, the P content of the NH_4 and Na salts was raised to 31 and 28% P respectively, but there was a marked decrease in the protein-precipitating property, probably due to the conversion of the polymetaphosphate into pyrophosphate. This could be avoided as follows:

150 mg. of the Na salt (20% P_7) were dissolved in 20 ml. water, the solution brought to pH 2 with HNO_3 and treated with $Ba(NO_3)_2$. A precipitate appeared which was left overnight, then centrifuged and washed with water until the diphenylamine test for NO_3^- was negative. The precipitate was washed with ethanol and dried over $CaCl_2$. This yielded 90 mg. of a white substance, with 42·0% Ba and 18·34 % P, or 1 atom Ba per 1·93 atoms P; the theoretical ratio Ba:P required for metaphosphate is 1:2. The preparation contained 2·03% C. The supernatant fluid separated from this Ba salt was brought to pH 5 with NaOH. A second precipitate appeared which, after washing and drying, yielded 75 mg. of a substance containing 42·8% Ba and 11·6% P, i.e. 1 atom Ba per 1·19 atoms P; theoretical ratio for pyrophosphate is 1:1. In addition, the preparation contained 3·1% C.

The assumption, that the two substances obtained by fractionation with $Ba(NO_3)_2$ were the Ba salts of metaphosphoric and pyrophosphoric acid respectively, was further strengthened by the results obtained with pure salts of these two acids.

200 mg. crystalline Na pyrophosphate (27·8 mg. P) and 100 mg. of pure Na hexametaphosphate (30 mg. P) each dissolved in 20 ml. water were brought to pH 2 with nitric acid and treated with $Ba(NO_3)_2$. After 24 hr. there was no precipitate in the sample containing pyrophosphate, but there was a considerable precipitate in the other sample. This precipitate, after washing with water and ethanol, yielded 140 mg. of a substance containing 42·6% Ba, 18·8% P and 1·27% C, and showed a ratio Ba:P = 1:1·95, which corresponds closely to that required for pure $Ba(PO_3)_2$. The barium pyrophosphate could only be precipitated by raising the pH to 5. The precipitate thus obtained (160 mg.) contained 56·4% Ba, 13·5% P and 1·36% C, thus showing a ratio Ba:P = 1:1·06, which corresponds closely to that required for pure $Ba_2P_2O_7$.

This experiment made it clear that the two easily hydrolyzable phosphorus compounds isolated from the mycelium of *A. niger*, when treated with $Ba(NO_3)_2$ at pH 2 and 5, behave in the same manner as pure metaphosphoric and pyrophosphoric acids. The C content of the compounds isolated from the mould may be due to the presence of organic material associated with the phosphoric acids, or to the use in the purifications of carbon-containing substances such as trichloroacetic acid and ethanol. Indeed, when pure Na metaphosphate and pure Na pyrophosphate were subjected to the same precipitation procedure as the mould-phosphorus compounds, the analysis of their Ba salts showed 1·27 and 1·36% C, respectively. All preparations had a high water content, but usually drying temperatures above 100° have been avoided so as not to affect the linkages in the polymetaphosphate. The problem of the molecular size of the mould-metaphosphate has yet to be investigated. It has been found, however, that the compound dialyzes at pH 7 much more slowly than pyrophosphate but at about the same rate as hexametaphosphate.

Examination of animal and mushroom tissues. The method described above for the isolation of mould-metaphosphate was applied to animal tissues (skeletal muscle, brain) and to the cultivated mushroom, *Agaricus campestris*. The main steps were as follows.

The material was extracted with trichloroacetic acid; the extract treated with an equal volume of ethanol; the filtrate brought with NaOH to pH 5·5, and the precipitate centrifuged and dried. Muscle and brain, unlike the mould, yielded preparations in which P_7 did not equal $P_{tot.}$. The product obtained from the muscle was identified as a mixture of Ca and Mg salts of adenosinetriphosphoric acid (ATP). From the brain a preparation was obtained which consisted of both easily and difficultly hydrolyzable phosphorus compounds. Both the muscle and the brain product exhibited the protein-precipitating property but only to a slight extent, 5–10 mg. of each of them being required for a positive test. The mushrooms, like the mould but unlike the animal tissues, yielded a preparation in which P_7 equalled $P_{tot.}$; 3 mg. of this substance were required for a positive protein precipitation test.

Differentiation between pyrophosphate and metaphosphate by means of enzymic reactions

Metaphosphate can be distinguished from pyrophosphate by its different behaviour towards certain enzymes.

Succinic oxidase. While 0·02 N-pyrophosphate abolishes the activity of succinic oxidase, metaphosphate, hexametaphosphate and ATP have little effect on the enzyme. Fig. 1 shows that metaphosphate isolated from *Aspergillus niger* as the Na salt behaves like pure metaphosphate. On the other hand, the other mould product which was isolated from the 'Ba pyrophosphate fraction' and identified as pyrophosphate had the same inhibitory effect on succinic oxidase as pure pyrophosphate.

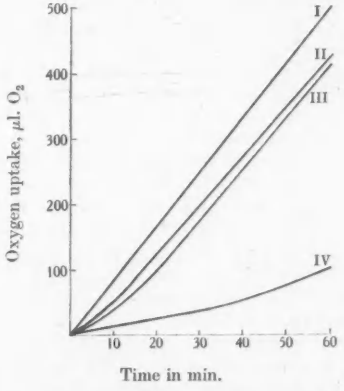


Fig. 1. Activity of succinic oxidase in presence of I, water; II, 0·02 N-Na metaphosphate; III, 0·02 N-mould metaphosphate; IV, 0·02 N-Na pyrophosphate. In each manometer vessel: 0·2 ml. enzyme + 0·2 ml. cytochrome *c* + 0·2 ml. M-phosphate buffer pH 7·3 + 0·25 ml. 0·25 M-Na succinate + 2 ml. tested compound.

Pyrophosphatase (yeast). This enzyme acts equally well on pure pyrophosphate and on pyrophosphate obtained from moulds. It does not attack metaphosphate, and its slight activity upon the mould-metaphosphate was due to an admixture of pyrophosphate (Table 2).

Table 2. *Action of pyrophosphatase on pyrophosphate and metaphosphate*

Substrate (Na salts; 0·4 mg. P)	Incubation at 30° and pH 7 (min.)	Dephosphorylation (%)
Pyrophosphate	30	66
	120	100
Metaphosphate	180	8
Hexametaphosphate	180	11
Crude preparation of mould metaphosphate	30	29
	180	33
Purified mould metaphosphate	180	10
Mould pyrophosphate	30	71

Metaphosphatase. This enzyme, present in *A. niger* (Mann, 1944), was found to act on metaphosphate as well as on pyrophosphate. Table 3 illustrates its activity towards pure substances as well as the mould products.

Table 3. *Metaphosphatase activity in the* A. niger *mycelium pulp*

Substrate (Na salts; 1 mg. P; 5 ml.)	Incubation 30° (hr.)	Dephosphorylation (%)
Pyrophosphate	2	40
	18	100
Metaphosphate	2	43
	18	100
Hexametaphosphate	2	39
	18	94
Crude preparation of mould metaphosphate	2	37
	18	90

Separation of the difficultly hydrolyzable phosphorus compound

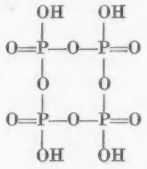
The 'Pb fraction II' obtained from the trichloroacetic acid extract at pH 7 was decomposed with H_2S, the solution neutralized with $Ba(OH)_2$ and filtered. From the filtrate a Ba salt was obtained which contained 2 % P. It was soluble in water but insoluble in 80 % ethanol; in this respect it resembled the Embden ester and α-phosphoglycerol. After the removal of Ba and hydrolysis with N-HCl the following percentages of total phosphorus appeared as orthophosphate at the times indicated: at 0 min.—0 %; at 7 min.—13·4 %; at 30 min.—22·7 %; at 3 hr.—37·5 %.

DISCUSSION

One of the main points which this investigation shows is that the mycelium of *A. niger* absorbs and converts a large proportion of orthophosphate into metaphosphate and pyrophosphate.

The occurrence of pyrophosphate in nature has been known for some time. It was isolated from muscle by Lohmann (1928), but was later shown to occur as a complex with adenylic acid. The presence or absence of *free* pyrophosphate in muscle was the subject of a controversy between Ferdmann & Dmitrenko (1933), Ferdmann & Feinschmidt (1935*a*) and Ferdmann & Feinschmidt (1935*b*) on one side and Lohmann & Schuster (1934), Parnas (1937) and Umschweif & Gibayło (1937*a*) on the other side. The two last-mentioned authors, using a technique by which it was possible to detect minute quantities of pyrophosphate in presence of orthophosphate, failed to detect any free pyrophosphate in muscle extracts. In fact, Ferdmann himself pointed out that he was not able in all his experiments to demonstrate free pyrophosphate in muscle. Of great interest is an observation by Cori (1942) who, using dialyzed rat-liver dispersions, noticed that the oxidation of pyruvate, succinate and glutamate was linked with the uptake and conversion of orthophosphate to pyrophosphate.

The isolation in pure form of metaphosphate from a natural source has not been reported previously. Albers (1940), in his review on phosphatases, remarked that, although a metaphosphatase is known to occur, so far no substrate had been found in nature which exhibited the properties of metaphosphate. A compound has now been isolated from *A. niger* which behaves like metaphosphate. It is decomposed by the mould metaphosphatase but is not attacked by the pyrophosphatase. It forms the typical Na, NH_4 and Ba salts of metaphosphoric acid and it acts as a protein-precipitating agent quantitatively in the same manner as pure metaphosphoric acid. However, it tends to form pyrophosphate when subjected to certain chemical procedures, and its rate of dialysis is slower than might be expected. It is conceivable that the mould-metaphosphate exists in the form of a polymetaphosphate of the type described by Graham (1834), Maddrell (1847), Fleitmann (1849) and Tammann (1892), which can be obtained by melting orthophosphate. It now appears that such substances actually occur in nature. With regard to animal tissues, fractions have been obtained which exhibit some protein-precipitating property, but they also contain a high percentage of adenosine polyphosphate which itself may be responsible for the precipitation phenomenon. There is some evidence that metaphosphate-like compounds are present in yeast. Lutwak-Mann & Mann (1935) noticed that only a part of the easily acid-hydrolyzable phosphate extracted by trichloroacetic acid from fresh bottom yeast occurred in combination with adenylic acid; the rest was composed of another phosphorus compound which was converted to orthophosphate after 7 min. acid hydrolysis, but was not a derivative of adenylic acid. Later, Umschweif & Gibayło (1937 *a*, *b*) isolated from yeast two distinct Ba salts, one of which they identified as Ba pyrophosphate and the other as the Ba salt of a peculiar polyphosphate which, according to the authors, was a tetraphosphate derived from diadenosinehexaphosphate. The metaphosphate obtained from moulds and this yeast compound show similar behaviour towards Ba- and Pb-nitrate. Should the mould compound be a tetraphosphate, its structure could be visualized as



So far, the size of the polyphosphoric acid molecule in the mycelium itself is unknown. Further study is necessary to establish whether it occurs as such or in combination with some organic material. It should be pointed out, however, that even the crude preparations of the mould metaphosphate contain very little nitrogen and pentose and cannot therefore be regarded as adenyl derivatives. On the other hand, it was frequently noted that the content of soluble polysaccharide in the mycelial extracts runs parallel to the content of the meta- and pyrophosphate. It may also be significant that crude preparations of meta- and pyrophosphate from *A. niger* always contained considerable amounts of Mg and Ca. Both these cations have also been found in crude preparations of adenosinetriphosphate isolated from skeletal muscles.

The ratio of metaphosphate to pyrophosphate was found to vary from one preparation to another. It is possible that the pyrophosphate is not originally present in the mycelium but that it is formed from the polymetaphosphate in the course of the isolation procedure.

SUMMARY

1. Two easily acid-hydrolyzable phosphorus compounds have been isolated in pure form from the mycelia of *Aspergillus niger*. One was identified as pyrophosphoric acid and obtained as crystalline Na pyrophosphate. The other was identified as metaphosphoric acid and obtained as Na, NH_4 and Ba metaphosphates.

2. Chemical and enzymic methods have been developed which, together with the protein precipitation test, make it possible to distinguish between pyrophosphoric acid and metaphosphoric acid.

3. The mycelium of *A. niger* also contains a difficultly acid-hydrolyzable phosphorus compound which has been obtained in the form of a water-soluble Ba salt.

I wish to thank Prof. D. Keilin, F.R.S., for his valuable criticism and kind interest in this work.

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The Effect of X-rays on the Respiration of Fowl Erythrocytes

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This paper deals with experiments concerning the effect of high doses of X-rays on the respiration of fowl erythrocytes.

METHODS

Preparation of material. Fowl erythrocytes were chosen for this study in view of their high O_2 consumption compared with non-nucleated erythrocytes. Six chicks aged 6–8 weeks were used alternately for all the present experiments. The blood was taken from the heart, defibrinated, filtered through sterilized glass wool and the filtrate centrifuged for 5 min. at 2500 r.p.m. The sediment of erythrocytes was then suspended in physiological saline (neutralized with 0·1 N-NaOH to phenolphthalein; Siebeck, 1923) and again centrifuged. This procedure was repeated three times in order to eliminate serum and leucocytes as completely as possible. After the third washing the sediment of erythrocytes was suspended in neutralized saline at a concentration of about 2×10^6 erythrocytes cu. mm. as estimated in a blood-counting cell.

Irradiation. The X-ray tube used in these experiments was operated at a tension of 35 kV. and a current of 15 mA. with a copper anticathode. The window was aluminium foil 30μ in thickness. Absorption analysis showed that the rays which penetrated through the window were mainly copper X-rays. The distance between the vessel in which the blood was irradiated and the focus was 50 mm. and the X-ray intensity was about 60,000 r./min. Irradiation was performed on 0·5 ml. portions of the erythrocyte suspension in flat-bottomed shallow glass dishes of 15 mm. diameter. The blood layer did not exceed 3 mm. in thickness.

Respiration. The respiration was measured in the Warburg apparatus at 39–40°. 1 ml. of suspension was used for each vessel for each estimation; three 0·5 ml. portions had therefore to be irradiated consecutively and afterwards combined. Parallel estimations of the respiration of non-irradiated (normal) erythrocytes of the same blood sample were carried out in every case. The portions to be irradiated, as well as those already irradiated, and controls were kept during the whole time of irradiation under equal conditions of temperature, etc. The first reading was made 20 min. after inserting the manometers in the water-bath (or about half an hour after the termination of irradiation). Readings were then taken, initially at intervals of 30 min. and later at intervals of 60 min.

Haemolysis. This was determined by the acid haematin method (Hawk & Bergeim, 1938). The erythrocyte suspension to be tested was centrifuged (5 min., 2500 r.p.m.), the supernatant solution was diluted (according to its haemoglobin content) with 0·1 N-HCl and the extinction of the resulting solution was determined photometrically. The percentage haemolysis was calculated from the haemolysis-grade/extinction curve which was obtained for every blood sample by the following method:

0·1 ml. of the whole erythrocyte suspension was haemolyzed with 9·9 ml. 0·1 N-HCl, and several known dilutions of the haemolysate in 0·1 N-HCl were measured photometrically. The curve obtained by plotting the extinction against haemolysate concentration formed a straight line at this level of concentration.

RESULTS

Three doses of X-rays were examined: 500,000, 1,000,000 and 2,000,000 r. The results of typical experiments are presented in Table 1.

Table 1. *Influence of irradiation on O_2 consumption of erythrocytes*

Irradiation dose (r.)	μl. O_2 consumed by 1 ml. erythrocyte suspension after (min.)										
	30	60	90	120	150	180	210	270	300	330	360
0	6·3	15·4	24·5	—	32·9	—	44·1	56·0	—	70·7	77·0
500,000	14·0	26·6	36·5	—	44·9	—	56·8	70·1	—	86·2	92·5
0	11·3	21·1	33·7	43·5	53·3	60·8	67·1	81·5	86·5	—	—
1,000,000	25·4	43·4	60·1	72·1	83·4	91·4	96·0	105·5	108·5	—	—
0	18·9	33·4	44·7	60·5	73·7	85·0	—	104·5	—	126·5	134·1
2,000,000	35·5	52·2	56·9	69·6	81·0	84·3	—	95·0	—	102·4	106·4

The rate of respiration of normal erythrocytes in saline decreases with time. In Fig. 1 the value for the O_2 consumption rate of normal blood at any specified moment was taken as 100 (horizontal line). Values for irradiated erythrocytes at the same time points are rates relative to this unit. The curves thus show plots against time of the percentage increase or decrease of the respiration of irradiated erythrocytes in comparison with non-irradiated controls.

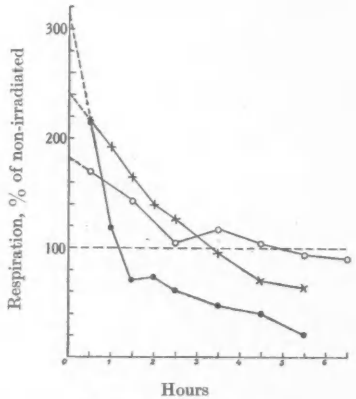

Fig. 1. Effect of X-radiation on the respiration of fowl erythrocytes. The data given are the O_2 consumption in the experimental vessels, expressed as a percentage of the O_2 consumption observed in the control vessels at a given time. ——— non-irradiated erythrocytes. —o— erythrocytes irradiated with 500,000 r. —×— erythrocytes irradiated with 1,000,000 r. —•— erythrocytes irradiated with 2,000,000 r.

Haemolysis. It is known that respiration is manifested by intact erythrocytes only, and not by their haemolysates. Effects due to haemolysis must therefore be taken into account.

The spontaneous haemolysis of a suspension of fowl erythrocytes in saline was found to be negligible even after 6 hr. of constant agitating at 39–40° (Table 2). Experiments showing positive results of haemolysis obtained with X-rays are very rare (Levin & Piffault, 1934, man, guinea-pig, with 1,200,000 r.); Halberstaedter & Goldhaber, 1943, 1944 (rabbit and fowl, with 2,000,000 r.).

Upon irradiation with doses of 500,000 and 1,000,000 r. no immediate haemolysis was observed. The supernatant in such cases is in fact paler than in non-irradiated controls; even with 2,000,000 r. immediate haemolysis did not exceed 4–5 %. However, blood incubated at 39–40° after irradiation begins to haemolyze. The speed of this haemolysis increases with the dose of X-rays for any given blood sample and varies from sample to sample; and with any particular sample it increases also with the storage time.

Table 2. *Haemolysis on incubation of irradiated and non-irradiated erythrocytes*

Irradiation dose (r.)	Haemolysis after incubation for (min.)	
	30 (%)	330 (%)
0	0·9	2·5
500,000	1·2	23·2
0	0·9	1·7
1,000,000	3·5	73·1
0	0·8	1·5
2,000,000	15·6	71·5

It should be noted that the post-irradiative course of haemolysis was not followed continuously for technical reasons. Haemolysis was determined on three 0·5 ml. portions which were irradiated consecutively, pooled (as in respiration experiments) and shaken at 39–40°. This quantity permitted only two haemolysis estimations. One was carried out after 30 min. incubation (corresponding to the first reading in the respiration experiments) and the other after 6 hr. incubation. The intermediate values of haemolysis were calculated by interpolation from these readings; for the purposes of this approximation the haemolysis-time curve was considered to be linear. The values representing averages of several experiments with blood of various chicks were used for the correction of the results of the respiration experiments presented in Fig. 1 (which are likewise averages). Correction of the data of each respiration curve by parallel values for haemolysis obtained with the same blood sample would obviously have

been preferable. However, since storage time seriously affects the haemolysis rate, correction on this basis was not in practice feasible. A corrected percentage respiration-increase/time curve is plotted in Fig. 2.

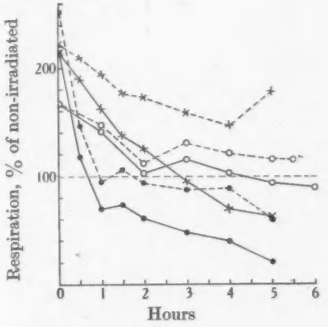

Fig. 2. Effect of X-radiation on the respiration of fowl erythrocytes, after correction for haemolysis. The data given are the O_2 consumption in the experimental vessels, expressed as a percentage of the O_2 consumption observed in the control vessels at a given time. ——— non-irradiated erythrocytes; —o— erythrocytes irradiated with 500,000 r.; ---o--- erythrocytes irradiated with 500,000 r., corrected for haemolysis; —×— erythrocytes irradiated with 1,000,000 r. ---×--- erythrocytes irradiated with 1,000,000 r., corrected for haemolysis; —•— erythrocytes irradiated with 2,000,000 r.; ---•--- erythrocytes irradiated with 2,000,000 r., corrected for haemolysis.

DISCUSSION

From the results in Table 1 and Fig. 1 it may be concluded that X-ray treatment of fowl erythrocytes in physiological saline stimulates their respiration to a high degree. The stimulation increases with the dose in the interval of 500,000–1,000,000 r.

The fact that the average stimulation effected by 2,000,000 r. is almost equal to that of 1,000,000 r. (Fig. 1) might be due to some destruction in the respiration system caused by the larger dose. In experiments on the effect of radiation on various cells, Wels (1924 *a*, *b*) found that the respiration of fowl erythrocytes was not stimulated by X-rays; however, the doses administered were very small in comparison to ours. Hazama (1924), on the other hand, who worked with γ-rays, found that irradiated fowl erythrocytes manifested a stimulated respiration and in this case the stimulation increased with the dose up to a certain limit above which it disappeared altogether.

As may be seen from Fig. 1 the stimulation is in all cases greatest at the beginning of the measurements, i.e. at about 30 min. from the termination of the radiation. In some instances stimulation records of 200–300% are obtained. The initial stimulation immediately after the termination of the irradiation may confidently be expected to be still higher. Its value can be approximately estimated by extrapolation of the curve to zero on the time co-ordinate (dotted lines of Fig. 1). As the respiration proceeds the stimulation curve falls off, the slope increasing with the irradiation dose administered. It intersects the horizontal curve in all cases and at later stages the respiration rate of irradiated cells seems to be lower than is that of the normal cells. Intersection occurs sooner with larger doses, with which both initial increase and subsequent decrease are more pronounced. For the case of 2,000,000 r. intersection also occurs in the total respiration-time curves (data of Table 1). This shows that the decrease, at least in this case, is not due to lack of substrate or accumulation of reaction product and, at least in part, must be due to haemolysis, the latter being almost negligible in the controls, but increasing in relation to the dose in the irradiated suspensions.

To arrive at the real stimulation of O_2 uptake by irradiation, the calculation must be based not on the initial number of cells irradiated, but, at every given moment, on that fraction which has escaped haemolysis. Correction of the values on this basis causes an increase in the general level of the stimulation values obtained. The increase, moreover, is especially marked for the later stages of the experiments when haemolysis is well advanced. For doses of 500,000 and 1,000,000 r., and most clearly for the latter, the correction raises the relative curves wholly above the level of the normal (horizontal curve of Fig. 2).

It is therefore suggested that X-ray treatment of fowl erythrocytes causes a stimulation of respiration rate which decreases slowly and can be noted as long as the cells escape haemolysis within the duration of our experiment. Integration of the curves within the interval of its terminal points is, of course, necessarily arbitrary. Discussion on this point is impossible, however, since only approximate haemolysis corrections have been available.

A dose of 1,000,000 r. is optimal for the stimulation of the respiration of fowl erythrocytes. At 2,000,000 r. the rays exert probably also a damaging effect upon the respiration system, since in this case inhibition in the later stages of respiration measurements remains manifest even after the introduction of the necessary corrections for haemolysis (Fig. 2).

Fowl erythrocytes thus constitute one of the few known examples in which irradiation causes a marked and persistent stimulation of a biological process. Stimulation of respiration by ray treatment has previously been observed by Stoklasa (1930) by β- and γ-rays in plants, and by Williams & Sheard (1932) for frog skin treated with fairly small X-ray doses.

SUMMARY

1. The effect of X-radiation on the respiration and haemolysis of fowl erythrocytes has been investigated.

2. It was found that the respiration of fowl erythrocytes is markedly stimulated by treatment with doses of 500,000–2,000,000 r.

3. The stimulation increases with the dose up to 1,000,000 r. The initial effect of 2,000,000 r. does not exceed that of 1,000,000 r.

4. If radiation is administered in optimal doses the stimulation induced persists as long as the erythrocytes escape haemolysis within the duration of these experiments (6 hr.).

5. Fowl erythrocytes, incubated at 39–40° (with shaking) following irradiation, undergo progressive haemolysis, the velocity of which is proportional to the X-ray dose previously applied. The haemolysis of non-irradiated erythrocytes under equal conditions is negligible even after 6 hr. incubation.

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